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Anaphylaxis Following Ingestion of Aneurine Hydrochloride

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In the last few years, as in the case of penicillin, reports of hypersensitivity or anaphylactic reactions following parenteral use of vitamins have been recorded (Frankland, 1962). A case of total anaphylaxis following oral ingestion of 100 mg. tablet of aneurine hydrochloride (vitamin B₁₂) is reported here.

CASE REPORT

A female patient, aged 35 years, was admitted to K. M. Hospital on 22/2/67 at 10-15 A.M. with a history of ingestion of one tablet of aneurine hydrochloride (100 mg.) at 8-00 A.M. Within a few minutes of ingestion of the tablet, she developed a rash and itching all over the body. She complained of pain in the chest, dyspnoea and a choking sensation in the throat about an hour later. The relatives revealed the history of the patient having taken one tablet of aneurine hydrochloride daily for 15 days two months ago and thereafter she had taken one tablet on the morning of admission. On examination, the patient was cold and clammy. Her pulse was 80/minute, blood pressure was 70/40 mm. Hg, respiratory rate was 30/minute. The skin revealed an erythematous petechial rash all over, the petechiae ranging in size from a pin point to a pin head. On systemic examination, she was fully conscious and answered questions relevantly. Examination of the respiratory system, alimentary system and central nervous system did not reveal any abnormality.

Laboratory investigations revealed that her haemoglobin was 12 g. per cent and W.B.C. count 1,200/c.mm. The electrocardiogram revealed non-specific ST-T changes. The patient was treated with intravenous fluid infusions with noradrenaline; I.V. hydrocortisone 100 mg. 6 hourly, injection antazoline hydrochloride and injection adrenaline (1:1000) 1 ml. stat. The blood pressure was maintained around 120/80 mm. Hg. Oral antihistaminics were continued. On the following day at about 10 P.M. the patient suddenly became restless. She was given symptomatic treatment but in spite of all efforts she expired at about 10-45 P.M.

Post-mortem examination revealed depigmented patches ranging from a pin point to a pin head in size all over the skin. The lungs revealed marked pulmonary oedema. The examination of the heart revealed no infarction. There were minimal atheromatous changes in the coronary arteries. Histopathology revealed epidermal basal cell hyperplasia of the skin. The lungs showed dilated bronchioles. Intervening lung parenchyma showed fibrosis and infiltration with a large number of chronic inflammatory cells. One of the arteries showed evidence of a recent thrombus. The examination of the liver, brain, kidney, heart did not reveal any abnormality.

DISCUSSION

Aneurine hydrochloride, also called thiamine hydrochloride, is crystalline vitamin B₁. It forms part of a co-enzyme needed for further oxidation of pyruvate which is an intermediate product in the oxidation of glucose. Taken by mouth, aneurine in quite enormous doses is without harmful effect. Parenteral administration in animals has shown that toxic effects are produced only with doses of at least 1000 times the requirement.

In man, parenteral administration of aneurine is rarely warranted. Very occasionally, patients who have had aneurine parenterally on several occasions develop a hypersensitivity. In these rare cases, on further administration, an anaphylactic reaction may result.

All untoward reactions to drugs, however, are not due to allergy and such terms as 'idiosyncrasy', 'intolerance' and 'toxicity' are not necessarily synonymous with allergy. Pharmacologically, numerous untoward reactions may occur as a result of such complex mechanisms as interference with physiological activity of specific enzyme systems.

Allergic reactions, on the other hand, require the existence of hypersensitivity mechanism. In the case of a simple drug or chemical, it must be assumed that there is a partial degradation of the offending drug with transformation into a hapten which by conjugation becomes antigenic and thus capable of entering the necessary antigen-antibody relationship.

There have been isolated clinical reports of toxic reactions to parenteral administration of aneurine. These probably represent rare instances of hypersensitivity (Shure, 1961; Yodkin, 1959). In these cases reported in literature, there has been a history of having taken the drug previously so that sensitisation had occurred. Any injection can cause sensitisation and one knows from experience with penicillin injection that the more the number of penicillin injections given the greater the chances of subsequent sensitisation. Sensitisation to vitamin B₁ injection does occur, yet it is difficult to understand exactly which component of a vitamin tablet or injection has acted as allergen.

Oral vitamin B₁ might cause allergic reaction in the same way as does oral penicillin. To our knowledge, anaphylactic shock after tablet has not been reported though there are case reports of severe allergic reactions after parenteral administration of aneurine (Shure, *loc. cit.*). Oral administration of a multivitamin capsule containing 1 mg. of aneurine is reported to have caused a day-long headache but the administration of other vitamins, omitting aneurine, caused no reaction.

In the case reported here, there is little doubt that the patient had typical symptoms and signs of an anaphylactic reaction after the ingestion of vitamin B₁ tablet. Since there was the history of having taken fifteen tablets of B₁ before, the probability is that she was sensitised to the drug and developed anaphylactoid reaction.

SUMMARY

A case of fatal anaphylactoid reaction following ingestion of aneurine hydrochloride is reported and the mechanism of the same has been discussed.

ACKNOWLEDGMENT

We take the opportunity to thank Dr. S. V. Joshi, Dean, K.M. Hospital for allowing us the use of hospital records. Berin brand aneurine hydrochloride and antistine brand antazoline hydrochloride were used.

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Nutrition

METABOLISM OF 2-¹⁴C-THIAZOLE LABELED THIAMINE IN MAN.

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In order to study the metabolism of 2-¹⁴C-thiazole labeled thiamine in man, a young male subject was fed a controlled diet providing about 1.4 mg thiamine per day. After three days on this diet, he received an oral dose of 0.592 mg 2-¹⁴C-thiazole labeled thiamine (46.9 μ C). The subject consumed the same diet for seventeen days after the administration of radioactive thiamine, and the excretion of the radioactivity in urine, feces and respiratory air was studied. The daily urinary excretion of radioactivity for the ten days following the dose was: 9.7, 6.7, 2.9, 2.5, 2.2, 2.0, 1.4, 1.5, 1.3 and 1.6% of the dose, respectively. The total radioactivity excreted in feces during the three days after the ingestion of the ¹⁴C-thiamine, measured by combustion method, accounted for 3.8% of the dose. No measurable amount of radioactive CO₂ could be detected in respiratory air. The biological half-life of the vitamin, as calculated from the rate of excretion, was estimated to be about eighteen days. Fractionation of the desalted urine on an Amberlite CG-50 column gave a pattern similar to that obtained by Balaghi and Pearson for similarly labeled thiamine in the rat. (Fed. Proc., 24: 691. 1965)

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Metabolism of Physiological Doses of Thiazole-2-¹⁴C-labeled Thiamine by the Rat

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ABSTRACT The metabolism of thiazole-2-¹⁴C-labeled thiamine was studied in the rat in order to compare its metabolism to that of pyrimidine-¹⁴C-labeled thiamine. The catabolic products of the thiazole-2-¹⁴C-labeled thiamine in the urine were separated by column and paper chromatographic techniques. More than 20 urinary metabolites were detected. The generation of ¹⁴CO₂ indicated breakdown of the thiazole moiety of thiamine. The marked similarity in the urinary chromatographic pattern found in this study to that obtained previously with pyrimidine-¹⁴C-labeled thiamine suggests that most of the urinary metabolites are derivatives of the entire thiamine molecule.

Recent studies of the catabolism of pyrimidine-¹⁴C-labeled thiamine have demonstrated the presence of a minimum of 22 different metabolites in rat urine (1). Of these metabolites only thiamine and 2-methyl-4-amino-5-pyrimidinecarboxylic acid have been identified (2). In an attempt further to elucidate the nature of the remaining unidentified thiamine metabolites, studies of the overall catabolism of thiazole-¹⁴C-labeled thiamine have been carried out and are presented in this report.

MATERIALS AND METHODS

Adult female rats of the Sprague-Dawley strain were used throughout the experiment. Housing of the animals, collection of urine and feces, and the composition of the thiamine-deficient diet have been described previously (3). Thiazole-2-¹⁴C-labeled thiamine was purchased from the Nuclear Chicago Corporation (specific activity 5600 cpm/μg). The purity of the compound was checked by column chromatography, paper chromatography, radioautography, thiochrome analysis and treatment of thiamine deficiency in rats.

The rats were depleted for 3 weeks with a thiamine-free diet and then intraperitoneal injections of radioactive thiamine were started. They were maintained for 4 weeks at each thiamine dosage level so that equilibrium was attained and the excretion of radioactivity in the urine, feces, and the respiratory CO₂ was studied. Daily

thiamine intake levels of 30 μg, 50 μg and 100 μg were studied.

At the end of each 24-hour collection period, the urine was pooled and an aliquot was taken for liquid scintillation counting. Feces samples were homogenized with 10 volumes of 0.2 N sulfuric acid in a Waring Blendor. The homogenate was steamed in an autoclave for 30 minutes and centrifuged for 45 minutes at 10,000 rev/min. The precipitate was resuspended in another volume of acid and re-extracted in a similar manner. The combined supernatant solutions were stirred for 30 minutes and duplicate samples were taken for scintillation counting. For respiratory studies, the rats were placed in a respiration chamber and the expired carbon dioxide was collected by drawing the expired air first through a column of Drierite and then through 2 successive cylinders, each containing 300 ml of a mixture of ethanolamine and ethylene glycol monomethyl ether (1:3 by volume) as suggested by Jeffay and Alvarez (4). At the end of each experiment, the ethanolamine trapping mixtures were combined and stirred for 30 minutes, and

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³ A portion of these data was taken from a thesis submitted by Mesbaheddin Balaghi to the faculty of the graduate school of Vanderbilt University in partial fulfillment of the requirement for the degree of Doctor of Philosophy in Biochemistry.

duplicate samples were taken for scintillation counting.

The radioactive metabolites in urine were purified by adsorption upon acid-washed charcoal and elution with a mixture of pyridine/ethanol/water (10/45/45 by volume). Eighty-five per cent of the radioactivity was adsorbed on charcoal and 75% of the adsorbed radioactivity was eluted by this method. The eluate was then subjected to chromatography on Amberlite CG-50 resin. Each peak obtained from this column was further resolved by ascending paper chromatography and the radioactive bands were localized by radioautography. The details of these procedures have been described by Neal and Pearson (1).

For determination of radioactivity in the fractions collected from ion exchange chromatography, 0.5 ml of each fraction was placed on an aluminum planchet, and 1 ml of ethanol was added. The planchets were then dried and counted in a Nuclear Chicago D-47 gas-flow counter equipped with a Model M5 sample changer. The radioactivity of the urine samples, feces extracts, and the pooled peaks obtained from column chromatography of urine were determined by liquid scintillation counting. The scintillation fluid used consisted of a mixture of: naphthalene, 100 g; PPO (2,5-diphenyloxazole) 7 g; POPOP (1, 4-bis-2-(5-phenyloxazolyl)-benzene) 0.3 g; and sufficient *p*-dioxane to make the volume up to one liter. The scintillation fluid used for counting the ethanolamine CO₂ trapping mixture was a 2:1 mixture of toluene and ethylene glycol monomethyl ether which contained 5.5 g of PPO/liter. In each case 10 ml of scintillation fluid were used per ml of sample and the internal recovery technique was used for correction of quenching.

Bioautography with the medium of Deibel et al. (5) was used to determine the biological activity of radioactive compounds found in urine. For this purpose 200 ml of single strength *Lactobacillus viridescens* thiamine assay medium⁴ containing 2% agar was autoclaved, cooled to 45°, seeded with a washed suspension of a 24-hour-old culture of *L. viridescens*, and poured into a rectangular glass dish.

When the medium solidified, the entire paper chromatograph or the excised bands containing the radioactive metabolites were put on the surface of the plate for 1 to 5 minutes. The paper was then removed and a layer of 400 ml of sterile 2% agar containing 25 ml of a 2% aqueous solution of 2,3,5-triphenyltetrazolium chloride was poured on the plate. When the second layer solidified, the plate was covered and incubated at 30° for 24 hours. The growth areas appeared as diffuse red spots against a yellow background. It was possible to detect the biological activity of 0.1 µg of thiamine by this method.

RESULTS

When the injection of thiazole-¹⁴C-labeled thiamine at a level of 30 µg daily was initiated in the thiamine-depleted rats, only 3.1% of the injected radioactivity appeared in the urine during the first 24 hours. This percentage increased slowly day by day and reached 43.7% on the twelfth day of injection. As the dosage of thiamine increased, a larger percentage of the radioactivity appeared in the urine. In table 1 it is shown that the urinary excretion of radioactivity accounts for 29 to 42% of that injected when the thiamine intake is between 30 and 100 µg/day. The rather large standard deviations observed were due in part to the difficulty of obtaining complete urine collections.

Analysis of the feces collected during a 9-week period from 16 rats maintained with 30 µg of ¹⁴C-thiamine/day yielded an average daily fecal excretion of 17.5% of the dose. As in the case of urine, the radioactivity of the feces increased grad-

TABLE 1
Urinary excretion of ¹⁴C-labeled thiamine compounds, expressed as percentage of the dose of radioactive thiamine¹

Dose	Excreted in urine
µg	%
30	29.18 ± 6.70 ²
50	38.29 ± 5.45
100	42.00 ± 10.08

¹ Each figure represents the mean daily excretion of 16 rats for 4-week periods (30 and 50 µg) or one-week period (100 µg).

² sd.

⁴ Difco Laboratories, Detroit, Michigan.

TABLE 2
Excretion of radioactive CO_2 by rats receiving various dosage levels of thiazole- ^{14}C -labeled thiamine

30 $\mu\text{g}/\text{day}$		50 $\mu\text{g}/\text{day}$		100 $\mu\text{g}/\text{day}$	
Total $^{14}\text{CO}_2^1$	% of dose	Total $^{14}\text{CO}_2^1$	% of dose	Total $^{14}\text{CO}_2^1$	% of dose
7.0	23.5	7.3	14.6	7.2	7.2
5.4	18.3	6.8	13.6	7.8	7.8
8.1	27.0	6.6	13.2		
8.1	27.0	6.5	13.0		
7.2	24.2				
7.1 ± 1.1^2	24.0 ± 3.61	6.8 ± 0.35	13.6 ± 1.24	7.5 ± 0.42	7.5 ± 0.42

¹ Each value represents the $^{14}\text{CO}_2$ excreted in a 24-hour period by a single rat expressed as micrograms of injected thiamine or as percentage of the dose. The values at the 30- and 50- μg dosage levels represent 24-hour collections but those at the 100- μg dosage level are extrapolations of 8-hour collections.

² Mean \pm SD.

ually after initiation of the injections and only 11.8% of the intake appeared in the feces during the first week.

Table 2 shows the results of the respiratory studies. These were carried out at various times during the fifth and sixth weeks after initiation of the particular dosage in question. With thiamine intakes of 30, 50 and 100 $\mu\text{g}/\text{day}$, 24.0, 13.6 and 7.5% of the injected dose is excreted as radioactive carbon dioxide, respectively. The absolute excretion of $^{14}\text{CO}_2$ was relatively constant at the 3 levels of intake.

To study the rate of turnover of the urinary thiamine compounds, 6 rats were depleted for a period of 3 weeks by feeding a thiamine-deficient diet. The animals then received daily injections of 30 μg thiazole- ^{14}C -labeled thiamine for 3 weeks. At the end of this period the injection of ^{14}C -thiamine was stopped and daily injections of 30 μg of unlabeled thiamine were begun. The excretion of radioactivity in the urine was studied over the 32-day period starting with the last injection of the ^{14}C -thiamine. Figure 1 shows that the rate of loss is a hyperbolic function with a half-life of about 9 days. The effect of thiamine deficiency on the urinary excretion of these compounds was studied in another group of rats whose body thiamine stores were labeled by daily injections of 30 μg of thiazole- ^{14}C -labeled thiamine. Thiamine deficiency was then induced by discontinuing the thiamine injections. Measurement of the radioac-

tivity in the urine of these animals gave a curve similar to that in figure 1, but the early part of the curve was steeper and the half-life of the radioactive components was about 6 days, 33% shorter than in the case of thiamine-sufficient rats.

A typical Amberlite CG-50 elution pattern of the radioactive metabolites of thiazole- ^{14}C -labeled thiamine is shown in figure 2. In most experiments between 75 to 90% of the radioactivity placed on

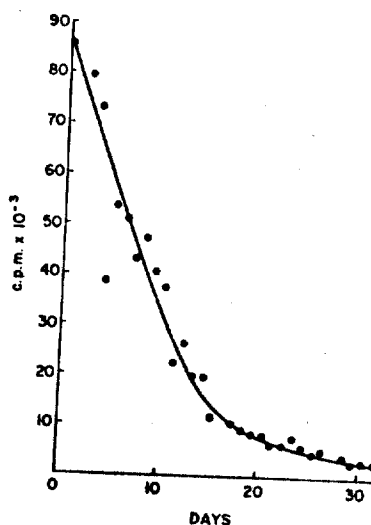


Fig. 1 The daily urinary radioactivity of rats receiving 30 μg of thiazole- ^{14}C -labeled thiamine/day for 3 weeks when transferred to 30 μg of unlabeled thiamine/day. Each point represents the total radioactivity excreted in the 24-hour urine samples of 6 rats.

the column was recovered by liquid scintillation counting. The radioactive metabolites were resolved into 5 peaks. Table 3 shows the percentage of total radioactivity residing in each of the 5 peaks at the 30- and 50- μ g levels of intake. More than 50% of the total radioactivity appears in the third peak. Peak IV (which contains thiamine) was found to contain an increasing percentage of the total radioactivity as the intake is increased.

Since it was known that the main component of peak I is a pyrimidine carboxylic acid (3) and since urinary pigments render studies of this peak difficult, this peak was not studied further. Peaks II, III, IV, and V were reduced in volume

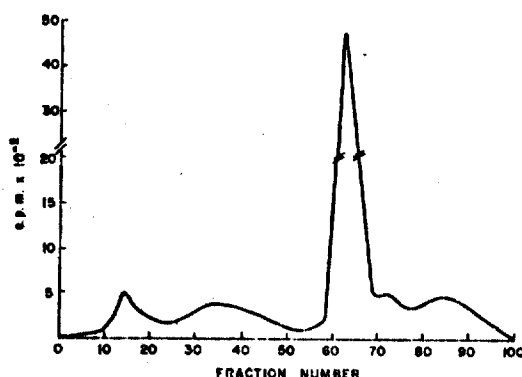


Fig. 2 Column chromatography of desalted rat urine on Amberlite CG-50 (200-400 mesh; H⁺ form, 1 \times 40 cm column). Flow rate 60 ml/hour, fraction size, 5 ml. The activity in counts per minute as determined by gas flow counting is that of 0.50-ml aliquots of the 5-ml fractions.

TABLE 3

Distribution of radioactivity among the 5 peaks obtained from Amberlite CG-50 chromatography of desalted urine from rats receiving 30 or 50 μ g of thiazole-¹⁴C-labeled thiamine day

Peak no.	Daily intake of thiamine ¹	
	30 μ g/day	50 μ g/day
	% of total urinary radioactivity	
I	7.0 \pm 1.2 ²	9.3 \pm 3.3
II	12.9 \pm 1.0	10.7 \pm 1.2
III	63.2 \pm 1.7	53.2 \pm 4.9
IV	5.6 \pm 0.9	19.9 \pm 5.9
V	11.0 \pm 1.7	6.5 \pm 5.9

¹ The values at the 30 μ g intake level are the average of 5 experiments, each experiment representing the metabolites from a pooled one-week urine collected from 16 rats. The values at the 50- μ g intake levels are the average of 8 such experiments.

² S.D.

under vacuum at 40°, lyophilized, and dissolved in 0.5 ml of water. Ascending chromatography was then carried out for 18 hours on Whatman no. 40 filter paper in n-propanol/water/1 M acetate buffer pH 5.0 (70/20/10 by volume). The dried paper was exposed to Eastman No-Screen x-ray film for 5 to 30 days depending upon the radioactivity of the sample. Figure 3 shows a schematic representation of the radioautograms with the compounds of quantitative importance being accented with thick lines. The mean R_f values of the 10 radioactive metabolites found by this method are also shown. These values varied considerably from one chromatogram to another because of the variable amounts of brown urinary pigments that were present. The latter appeared in rather large amounts principally in peaks II and III.

After the compounds were located on the paper by radioautography, their bio-

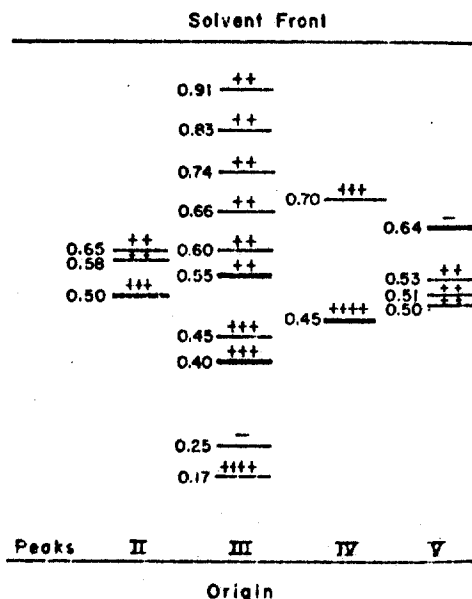


Fig. 3 Schematic drawing of radioautograms of radioactive peaks seen in figure 2. Solvent n-propanol/water/1 M acetate buffer pH 5.0 (70/20/10 by volume). Ascending technique for 18 hours. Chromatograms were exposed to the x-ray film for 10 days. The thick lines represent the main components of each peak. The mean R_f values are shown to the right of each band and the biological activity for *L. viridescens* is shown on the left. (+++ extensive growth; ++ good growth; + slight growth, - no growth)

logical activity was checked by bioautography. The results of these studies are also shown in arbitrary growth units in figure 3. All of the 10 metabolites except the metabolite with the R_f of 0.25 in peak III and the metabolite of R_f 0.64 in peak V showed traces of biological activity for *L. viridescens*. Tube assay of the urine, however, indicated that the percentage of microbiological activity was no more than 1% of that found by measurement of radioactivity.

DISCUSSION

As the dosage of thiamine was increased, the amount of radioactivity appearing in the urine also increased (table 1). For example, when the daily thiamine intake was increased from 30 to 100 μg the amount of the radioactivity appearing in urine increased from 29 to 42% of intake. Under these conditions the metabolite in urine that increased disproportionately with the dosage was thiamine itself (table 3), i.e., when the daily thiamine intake was increased from 30 to 50 μg , the percentage of total urinary radioactivity in peak 4 (which contains thiamine) increased 3 times (from 5.6 to 16.9%). This observation agrees with other data reported from this laboratory (8) and is consistent with the concept that urinary free thiamine represents an amount in excess of the minimal physiological needs of the animal.

Iacono and Johnson (8) reported that 16 urinary metabolites of thiazole- ^{14}C -labeled thiamine were excreted by the rat. In this study using a more elaborate separation procedure we have detected 19 in addition to those present in peak I of the Amberlite CG-80 column. Had the latter been considered, our total would have exceeded 30, i.e., comparable to the number found in our previous studies with pyrimidine ^{14}C -labeled thiamine (1). The data of Iacono and Johnson (8) also indicate that three of their radioactive bands contained the bulk of the radioactivity. This is in reasonable agreement with our observation of 3 major radioactive metabolites because it is likely that their major radioactive bands contained more than one compound.

The breakdown of the thiazole ring as evidenced by the generation of $^{14}\text{CO}_2$ is in accord with other reports in the literature. Borscock et al. (7) studied the metabolism of ^{35}S -labeled thiamine in man and found up to 25% of the intake as inorganic sulfate in the urine. Iacono and Johnson (8) recovered 0.2 to 3.7% of a 1-mg dose of thiazole- ^{14}C -labeled thiamine as respiratory $^{14}\text{CO}_2$. The quantitative aspects of the data reported by these 2 groups of workers cannot, however, be compared with those recorded here since their data were obtained in acute studies, whereas ours were derived from long-term studies at physiological levels of thiamine intake. In these studies, with daily thiamine intakes of 30, 50 and 100 μg , 24%, 13.6% and 7.5% of the radioactivity was excreted as $^{14}\text{CO}_2$, and the absolute values for radioactive CO_2 excreted were equivalent to 7.1, 6.8 and 7.5 μg of thiamine, respectively.

These data indicate that the thiazole ring of thiamine is considerably more labile than when it exists as the free thiazole moiety. The latter is quite stable metabolically as shown by the experiments of Ismail et al. (9). These workers injected ^{35}S -labeled 4-methyl-5-(2 hydroxyethyl) thiazole into rats and recovered more than 98% of the dose as 4-methylthiazole-5-acetic acid, a derivative of the intact thiazole ring. This stability difference can be demonstrated in vitro as well, i.e., a pH of 8.2 is sufficient to open the thiazole ring of thiamine to give a thiol form which readily oxidizes to the disulfide in air at room temperature. Under similar conditions, the free thiazole molecule remains intact.

According to currently accepted theory, the carbon in the 2-position of the thiazole ring is the active metabolic site of the thiamine molecule. Because, in our studies, the absolute amount of thiamine destroyed by oxidation of this carbon atom to $^{14}\text{CO}_2$ approximates the minimum thiamine requirement of the rat (10) (7-8 μg) it is tempting to suggest that this oxidative loss may be a measure of the "endogenous" thiamine utilization (minimal requirement?) of the rat. Our data suggest further that this oxidation may be independent of thiamine intake. Whether it is also independent of body stores cannot be

decided since no estimates of the latter were made.

The pattern of urinary radioactivity obtained by column chromatography on CG-50 (fig. 2) strongly resembles that reported by Neal and Pearson (1) in similar studies with pyrimidine-¹⁴C-labeled thiamine. Since the pyrimidine ring is not broken down to form CO₂, it would be anticipated that a higher percentage of radioactivity from thiamine containing ¹⁴C in the pyrimidine ring would appear in urine than from thiazole-¹⁴C-labeled thiamine. This has actually been observed by Neal and Pearson (unpublished data) who recovered 76 to 81% of the injected radioactivity from the urine of rats that had received 100 µg of pyrimidine-labeled thiamine daily for a long period. In these studies which were carried out with thiazole-¹⁴C-labeled thiamine under essentially equivalent experimental conditions the recovery of radioactivity from urine was only about 40 to 45%.

The marked similarities of the urinary chromatographic pattern obtained with both thiazole-¹⁴C- and pyrimidine-¹⁴C-labeled thiamine suggest that the large number of thiamine metabolites found in urine by Neal and Pearson (1) may be derivatives of the entire thiamine molecule rather than metabolic products of its pyrimidine or thiazole moieties. Furthermore, the marked lability of the thiazole ring observed in this study makes it highly unlikely that free thiazole or its derivatives occur in any quantity in rat urine.

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Nutrition

**INTRACELLULAR DISTRIBUTION OF RADIOACTIVE
THIAMINE IN NORMAL AND THIAMINE DEFICIENT RATS.**
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Distribution of radioactive thiamine in intracellular fractions of liver, brain, kidney and testes was studied in adult rats maintained on a physiological thiamine intake. In all tissues 60-70% of the radioactivity was found in the mitochondrial plus soluble fractions. About 20% was present in the nuclear fractions from liver, kidney and testes but only 9% was found in brain. Liver and kidney microsomal fractions contained 7-8% of the radioactivity but 20-24% in brain and testes appeared in this fraction. During thiamine depletion the radioactivity of the intracellular fractions of all tissues depleted at the same rate i. e. no one thiamine compartment is preferentially maintained. This suggests that transketolase (the enzyme most sensitive to thiamine deficiency) has a higher thiamine requirement than other thiamine-requiring enzymes or that the apoenzyme level is reduced. (Supported by Grant AM-07709 from USPHS.)

From the Medical University Clinic, Erlangen-Nürnberg
(Director: Prof. Dr. W. LENNING) and the Wilhelm-Conrad
X-ray Clinic, Giessen University (Director: Prof. Dr. G. BARTH).

Gunther BARTH, Eric DISTER and Herbert GRAEBNER

ADDITIONAL DRUG THERAPY OF THE TUMOR BED

Report IV: investigations concerning the problem
of the paratumoral effect of thiamine on the
regression of the experimental sarcoma 180 and
the survival time of the homozygous mouse strain
NMRI.

With 5 figures

It seemed expedient to include vitamin B as
well within the scope of present test series, in addition¹
to irradiation, when investigating the drug therapy
applied to the healthy tumor environment. Findings in
recent years indicated that hardly any reactions occurred
after irradiation therapy of malignant tumors in patients, follow-
ing thiamine and pyridoxine doses. As early as 1942
BOTSZTEJN (5) and LUDIN (6) obtained good results during
radiation syndrome therapy by using vitamin B application.
In 90 % of the cases improvement could be achieved¹ regarding
complaints which occur in the course of the radiation
syndrome; therefore the initiated irradiation therapy could
be completed without interruptions. Since only very few data
are available so far in literature on the application of
thiamine and pyridoxine for tumor therapy, we investigated the
effect of these two compounds on the fast-growing sarcoma 180
(CROCKER-sarcoma) in an inbred mouse strain (see also the
following report).

The investigation is intended to clarify how far vitamins B₁ and B₆, administered in addition to irradiation therapy, promote tumor regression and prolong survival time.

Regarding vitamin B₁: it is considered proven today that said vitamin is involved as co-factor in essential intermediary metabolic processes. Carbohydrate metabolism should be mentioned in the first place: vitamin B₁ is correlated with the same. The vitamin B₁ requirement depends on the extent of the carbohydrate supply, since a diet rich in carbohydrates increases the thiamine need (7). As pyrophosphoric acid ester, thiamine constitutes the co-ferment of the carboxylase which develops at various points during carbohydrate decomposition. Its important task consists in the decarboxylation of the pyruvic acid which finally leads to the formation of the acetyl-coenzyme A; with the aid of the latter, oxalacetic acid is condensed with "activated" acetic acid into citric acid. This involves the transition from anaerobic to aerobic glycolysis which then occurs in the so-called citric acid cycle (8). Furthermore, carboxylase brings about the conversion of α -ketoglutaric acid into succinic acid. - However, pyruvic acid and α -ketoglutaric acid are generated on a larger scale when the organism is subjected to the effect of irradiation (9). Reduction of the alkali reserve would result, following the retention of these "slag-substances".

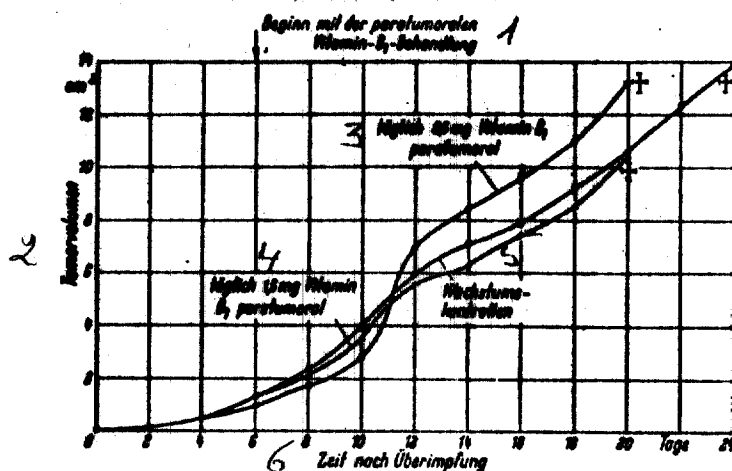


FIGURE 1. Tumor development with paratumoral administration of vitamin B₁ doses of differing strength, without X-ray irradiation.

- 1) Start of the paratumoral vitamin B₁-therapy.
- 2) Tumor volume
- 3) 0,5 mg vitamin B₁ daily, paratumorally
- 4) 1,5 mg vitamin B₁ daily, paratumorally
- 5) Growth controls
- 6) Time after implant.

Methodology

The experiments were performed in a total of 235 male albino mice of the inbred strain NMRI (Naval-Medical Research Institute). We used the histologically confirmed CROCKER-sarcoma of the mouse as test tumor, known under the definition S 180; it was implanted according to the method of OETTEL and WILHELM (10,11).

We irradiated under depth therapy conditions, using the method specified earlier (12). Initially, a preliminary test was performed on the 6th day after the tumor implant; this involved paratumoral vitamin B₁-treatment of the non-irradiated tumor.

To determine whether the effect on the non-irradiated tumor depends on dosage, we tested two differently strong Vitamin B₁ doses^{*)}, namely: 0,5 mg as well as 1,5 mg/day.

MAURER and DITTMAYER (13,14), who investigated the effect of ionizing radiation on vitamins, found that the vitamins are thereby more or less completely destroyed. To avoid the chemical-radiation alteration of vitamin B₁ in the organism of test animals subjected to X-rays, we always injected the preparation during the principal test following each irradiation only.

The investigations summarized in Figure 1 showed that with a daily administration of 0,5 mg thiamine, tumor growth accelerates starting from the 11th day after the implant as compared to the controls. The sarcomas in the animals treated with 1,5 mg vitamin B₁ daily showed less and slower growth, definitely "at variance" from the controls.

The death rate curve indicates that the animals initially lived longer with thiamine doses of 0,5 and 1,5 mg respectively; later, however, the death rate accelerated; one animal survived. The 1,5 mg vitamin B₁ dose had a more favorable effect on the tumor volume than the smaller dose; it was therefore selected for the main test (Figure 2).

*) "Benerva" of the German Hoffmann-La Roche Ltd. Co., Grenzach/Baden.

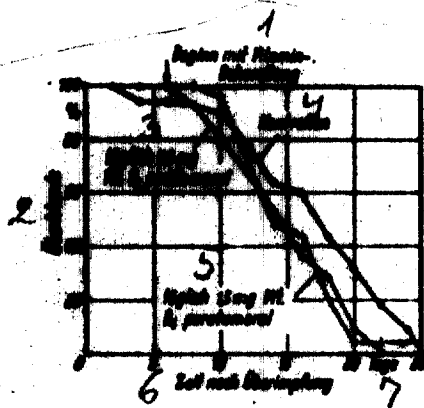


FIGURE 2. Death rate curves of the preliminary test.

- 1) Start of the vitamin therapy
- 2) Survivals
- 3) 0,5 mg Vitamin B₁ daily, paratumorally
- 4) Controls
- 5) 1,5 mg vitamin B₁ daily, paratumorally
- 6) time after implant.
- 7) days.

For subsequent X-ray irradiation, we selected the optimal individual dose of 510 R applied every second day, found earlier by BARTH, GRAEBNER and WACHSMANN (12), as tested in a heterozygous "Handler" strain. To eliminate injection stress as a possible error source when evaluating survival time, control animals received 0,2 ml of a physiologic common salt solution daily, applied paratumorally. Irradiation was started when the average tumor size reached the volume of 2 ml.

Result

With daily paratumoral administration of 1,5 mg thiamine, minor tumor volume increase became initially evident; from the 4th day after the start of the irradiation, however, the sarcoma 180 regressed continuously up to the last day of the irradiation. To discriminate between the local vitamin B₁ effect

with paratumoral application and the general effect of thiamine, we administered thiamine intramuscularly to a second animal group, i.e. not in the vicinity of tumors. In this case, the experimental tumor was much less strongly affected. (The substantial volume regression up to the 14th day after the start of the therapy is not the result of successful irradiation therapy; instead, it originates from the survival of a single animal with a very small tumor).

The tumor volume in the control group increased initially up to the very high average value on the 8th day after the start of the irradiation; but it continued to decrease subsequently up to the 14th day, to 2,8 ml. The sarcoma began to grow again up to the 18th day; then merely hesitant tumor regression was evident up to the 22. day (Figure 3).

The death rate curves are of specific interest here: as compared with controls which were subjected to irradiation only, the death rate curves, as those of the preliminary test, indicate a substantially accelerated death rate for the animals with paratumoral as well as with intramuscular administration of vitamin B.¹ Nevertheless, a significant drop of the survival rate is evident in the control group as well, since all animals had died by the 22nd day after the start of the irradiation. This is a significantly poorer result than obtained with similar tests in mice of a heterozygous strain (Figure 4).

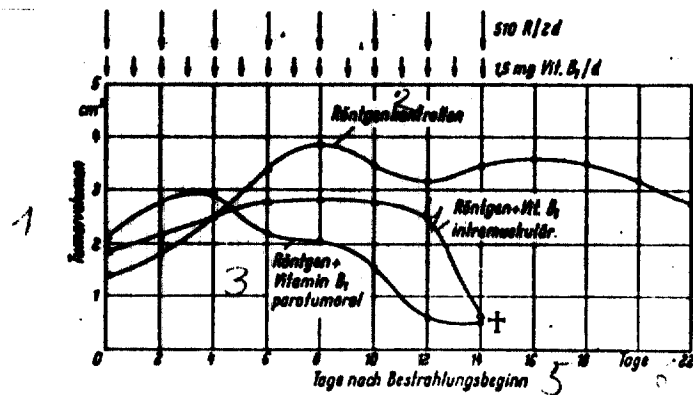


FIGURE 3. Tumor volume development in case of paratumoral application of vitamin B₁, as compared with the controls.

- 1) Tumor volume
- 2) X-ray controls
- 3) X-ray and vitamin B₁, paratumorally
- 4) X-ray and vitamin B₁, intramuscularly
- 5) Days after start of irradiation
- 6) days.

Discussion of the test results.

Under X-ray irradiation, the effect on tumors proved to be unsatisfactory in 100 mice of a homozigous strain with sarcoma 180. The number of test animals was kept intentionally high so as to obtain precise data on the effect of the irradiation in 48-hour cycles. The earlier death of the animals--none of them survived the 22nd day following the start of the irradiation--not only proves the existence of a correlation between tumor size, the applied high-volume dose and survival time, but also excludes any comparison with earlier results reported by us (1, 2,3,4).

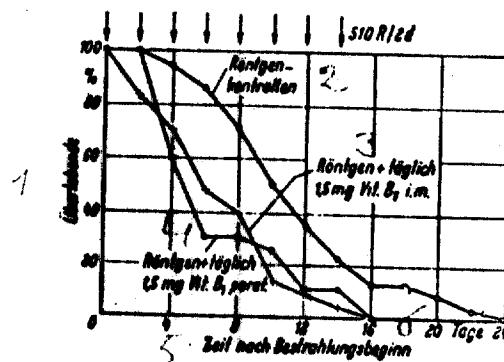


FIGURE 4. Earlier death of animals subject to parenteral vitamin B₃ administration and X-ray irradiation, as compared with the X-ray controls.

- 1) survivals
- 2) X-ray controls
- 3) X-ray and 1,5 mg vitamin B₃, i.m. daily.
- 4) X-ray daily; 1,5 mg vitamin B₃ paratumorally.
- 5) Time after start of irradiation;
6. days.

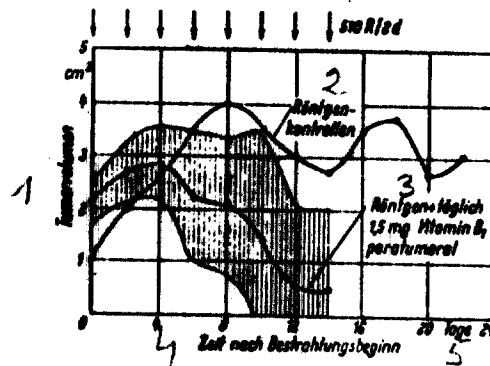


FIGURE 5. Significant difference in tumor volume development during X-ray irradiation and daily paratumoral administration of vitamin B₁ (shaded area = 3 x average error of the average value).

- 1) Tumor volume
- 2) X-ray controls
- 3) X-ray and daily 1,5 mg vitamin B₁, paratumorally
- 4) Time after start of irradiation
- 5) days.

Under the direct effect of thiamine on the tumor bed a significant difference in tumor volume development became evident; however, the general condition of the animals had deteriorated considerably as a consequence of frequent necroses, as the death rate curves indicate (Figure 5).

The results of this initial test series concerning the administration of vitamin B₁ during tumor irradiation lead to the conclusion that thiamine, presumably on the basis of its close correlation with carbohydrate metabolism, improves oxygen utilization in the paratumoral tissue on the one hand, but on the other hand also in the tumor cell itself.

As for the effect of vitamin B₁ on the survival rate in case of X-ray irradiation:

the results of other investigations are not very convincing either; for example: survival time tested by PFAB (16) in the course of irradiation involving the entire body of tadpoles exposed to an environment containing vitamin B₁, and the findings of RÖHRlich (16) who likewise tested vitamin B₁ in tadpoles under the effect of X-rays. Our findings demonstrate: the thiamine effect does not prolong the survival time of the animals in case of the fast-growing CROCKER sarcoma in mice of the NMRI inbred strain; instead, the survival time is, surprisingly, abbreviated. We are therefore of opinion that the effect of vitamin B₁ on the survival time of the animals and on the tumor volume should be investigated in heterozygous mouse strains as well, under identical test conditions, and we consider it necessary in general to undertake similar investigations, using other types of experimental tumors.

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Aus der Medizinischen Universitätsklinik Erlangen-Nürnberg (Direktor: Prof. Dr. N. Henning) und der Wilhelm-Conrad-Röntgen-Klinik der Universität Gießen (Direktor: Prof. Dr. Dr. G. Barth)

Zusätzliche medikamentöse Behandlung des Tumorbettes

IV. Mitteilung: Untersuchungen zur Frage der paratumoralen Wirkung des Thiamins auf die Rückbildung des experimentellen Sarkoms 180 und die Überlebenszeit des homozygoten Mäusestammes NMRI

Von

Gunther Barth, Erich Dister und Herbert Graebner

Mit 5 Abbildungen

Im Rahmen der in dieser Untersuchungsreihe zusätzlich zur Bestrahlung zu erprobenden medikamentösen Behandlung der gesunden Tumorumgebung [1, 2, 3, 4] schien es angeraten, auch das Vitamin B₁ einzusetzen. Erfahrungen der letzten Jahre zeigten, daß durch Gaben von Thiamin und Pyridoxin bei Patienten kaum Reaktionen nach Strahlenbehandlung bösartiger Tumoren auftraten. Durch Vitamin-B₁-Applikation erzielten schon 1942 *Botsztein* [5] und *Lidén* [6] bei der Therapie der Strahlenkrankheit gute Erfolge. In 90% der Fälle konnte Besserung der beim Strahlensyndrom auftretenden Beschwerden erreicht und so die begonnene Strahlentherapie ohne Unterbrechung zu Ende geführt werden. Da bisher nur sehr spärliche Angaben über die Anwendung von Thiamin und Pyridoxin zur Tumorbehandlung im Schrifttum vorliegen, untersuchten wir den Einfluß dieser beiden Verbindungen auf das schnell wachsende Sarkom 180 (Crocker-Sarkom) an einem Mäuse-Inzuchtstamm (s. auch nachfolgende Mitteilung). Es sollte dabei geklärt werden, in welchem Umfang die Vitamine B₁ und B₆ zusätzlich zur Strahlenbehandlung die Tumorregression fördern und die Überlebenszeit verlängern.

Für das Vitamin B₁ gilt heute als erwiesen, daß es als Co-Faktor an wesentlichen intermediären Stoffwechselprozessen beteiligt ist. In erster Linie ist der Kohlenhydratstoffwechsel zu nennen, mit dem es in Wechselbeziehung steht. Der Bedarf an Vitamin B₁ hängt vom Umfang der Kohlenhydratzufuhr ab, da kohlenhydratreiche Kost den Thiaminbedarf erhöht [7]. Als Pyrophosphorsäureester bildet Thiamin das Co-Ferment der Carboxylase, die an verschiedenen Stellen des Kohlenhydratabbaues ansetzt. Ihre wichtigste Aufgabe besteht in der Decarboxylierung der Brenztraubensäure, wodurch letztlich die Entstehung von Acetyl-Coenzym A vermittelt wird, mit dessen Hilfe die Kondensation von

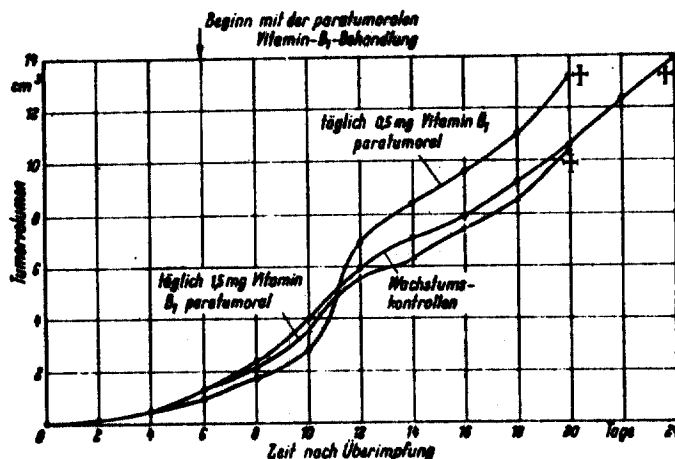


Abb. 1. Tumorentwicklung unter paratumoraler Verabreichung zweier verschieden hoher Vitamin-B₁-Dosen ohne Röntgenbestrahlung.

Oxallessigsäure mit „aktivierter“ Essigsäure zur Zitronensäure erfolgt. Damit erfolgt der Übergang von der anaeroben zur aeroben Glykolyse, die dann im sogenannten Zitronensäurezyklus abläuft [8]. Weiterhin bewirkt Carboxylase die Überführung der α -Keto-glutarsäure in die Bernsteinsäure. — Brenztraubensäure und α -Keto-glutarsäure werden aber gerade bei Strahleneinwirkung auf den Organismus in vermehrtem Umfang gebildet [9]. Verminderung der Alkalireserve durch Liegenbleiben dieser „Schlackensubstanzen“ wäre die Folge.

Methodik

Die Experimente erfolgten an insgesamt 235 männlichen weißen Mäusen des Inzuchtstammes NMRI (Naval Medical Research Institute). Als Versuchstumor verwendeten wir das unter der Bezeichnung S 180 bekannte histologisch gesicherte Crocker-Sarkom der Maus, das nach dem Verfahren von Oettel und Wilhelm [10, 11] überimpft wurde.

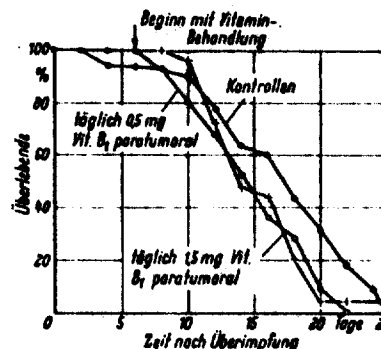
Wir bestrahlten unter Tiefentherapiebedingungen in der gleichen Weise, wie früher beschrieben [12]. Zunächst wurde in einem Vorversuch am 6. Tag nach der Tumortransplantation mit der paratumoralen Vitamin-B₁-Behandlung des unbestrahlten Tumors begonnen. Um abzugrenzen, ob die Beeinflussung des unbehandelten Tumors eine Dosisabhängigkeit zeigt, testeten wir zwei verschieden hohe Dosen von Vitamin-B₁^{*}, und zwar 0,5 mg sowie 1,5 mg/die.

Maurer und Dittmeyer [13, 14], die die Wirkung von ionisierenden Strahlen auf Vitamine untersuchten, stellten fest, daß diese dabei mehr oder weniger zerstört werden. Um eine strahlenchemische Veränderung des Vitamin B₁ im Organismus der Versuchstiere unter Röntgenbestrahlung zu vermeiden, injizierten wir daher im Hauptversuch das Präparat immer erst im Anschluß an jede Bestrahlung.

Die in Abbildung 1 zusammengefaßten Untersuchungen ergaben, daß unter täglicher Verabreichung von 0,5 mg Thiamin das Tumorstadium vom 11. Tag nach Überimpfung an gegenüber den Kontrollen beschleunigt ist. Bei den täglich

* Benerva der Deutschen Hoffmann-La Roche AG., Grenzach/Baden.

Abb. 2. Absterbekurven des Vorversuches.



mit 1,5 mg Vitamin B₁ behandelten Tieren zeigten die Sarkome geringeres, von den Kontrollen deutlich unterscheidbares langsames Anwachsen.

Aus der Absterbekurve geht hervor, daß bei Gabe von 0,5 bzw. 1,5 mg Thiamin die Tiere anfänglich länger lebten, dann jedoch schneller starben; ein Tier überlebte. Die Dosis von 1,5 mg Vitamin B₁ wirkte sich auf die Beeinflussung des Tumorummens günstiger aus als die geringere Dosis; sie wurde deshalb für den Hauptversuch gewählt (Abb. 2).

Bei den folgenden Röntgenbestrahlungsversuchen wählten wir die bereits von Barth, Graebner und Wachmann [12] gefundene optimale Einzeldosis von 510 R jeden zweiten Tag, wie sie an einem heterozygoten Hündlerstamm erprobt war. Um den Injektionsstoß als eventuelle Fehlerquelle in der Beurteilung der Überlebenszeit ausschalten zu können, wurde Kontrolltieren täglich 0,2 ml physiologische Kochsalzlösung paratumoral verabreicht. Die Bestrahlung begann bei einer durchschnittlichen Tumorgroße von 2 ml Volumen.

Ergebnis

Unter täglicher paratumoraler Verabreichung von 1,5 mg Thiamin zeigte sich anfänglich geringfügige Tumorummenzunahme, ab 4. Tag nach Bestrahlungsbeginn jedoch eine kontinuierliche Rückbildung des Sarkoms bis zum letzten Tag der Bestrahlung. Um die lokale Vitamin-B₁-Wirkung bei paratumoraler Applikation von einer Allgemeinwirkung des Thiamins abgrenzen zu können, verabreichten wir einer zweiten Tiergruppe Thiamin intramuskulär, d. h. tumorfrem. Hier zeigte sich eine wesentlich geringere Beeinflussung des Versuchstumors. (Der starke Volumrückgang bis zum 14. Tag nach Behandlungsbeginn beruht nicht auf einem Erfolg der Strahlentherapie, sondern auf dem Überleben eines Tieres mit sehr kleinem Tumor.)

Bei der Kontrollgruppe nahm das Tumorummen zunächst bis zu einem sehr hohen Durchschnittswert am 8. Tag nach Bestrahlungsbeginn zu, nahm dann aber bis zum 14. Tag kontinuierlich auf 2,8 ml ab. Nach weiterem Einsetzen erneuten Sarkomwachstums bis zum 18. Tag bildete sich der Tumor bis zum 22. Tag dann nur noch zögernd zurück (Abb. 3).

Von besonderem Interesse sind hier die Absterbekurven, die — ähnlich wie im Vorversuch — sowohl bei paratumoraler als auch bei intramuskulärer Verabreichung von Vitamin B₁ wesentlich rascheres Absterben der Tiere gegenüber

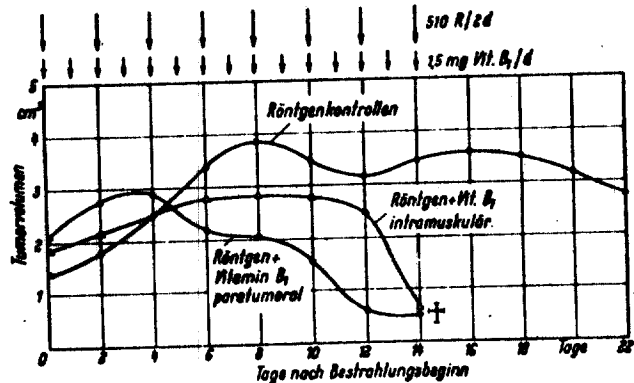


Abb. 3. Tumervolumenentwicklung bei paratumoraler bzw. intramuskulärer Verabreichung von Vitamin B₁ im Vergleich zu Kontrollen.

den Kontrollen, die nur bestrahlt wurden, erkennen lassen. Doch zeigt sich auch in der Kontrollgruppe eine sehr starke Abnahme in der Überlebensrate; denn bis zum 22. Tag nach Bestrahlungsbeginn waren bereits sämtliche Tiere gestorben. Dies ist gegenüber früheren Untersuchungen [12] ein signifikant schlechteres Ergebnis, als es bei gleichen Versuchen an Mäusen eines heterozygoten Stammes erzielt worden war (Abb. 4).

Diskussion der Versuchsergebnisse

Bei 100 Mäusen eines homozygoten Stammes mit Sarkom 180 wurden unter Röntgenbestrahlung die Tumoren nur unbefriedigend beeinflusst. Die Anzahl der Versuchstiere wurde absichtlich sehr hoch gewählt, um ein genaues Bild über die Auswirkung der Bestrahlung im 48-Stunden-Rhythmus zu erhalten. Das frühere Absterben der Tiere — keines überlebte den 22. Tag nach Bestrahlungsbeginn — beweist nicht nur das Bestehen einer Relation zwischen Tumorgroße, verabreichter hoher Raumdosis und Überlebenszeit, sondern läßt auch keinen Vergleich mit früheren von uns mitgeteilten Ergebnissen zu [1, 2, 3, 4].

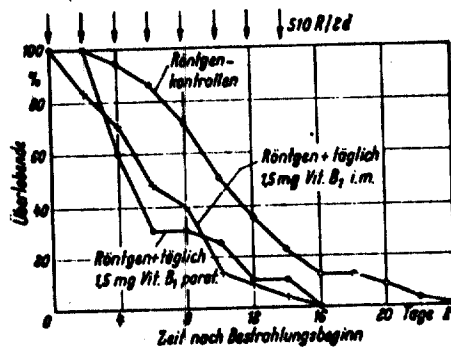
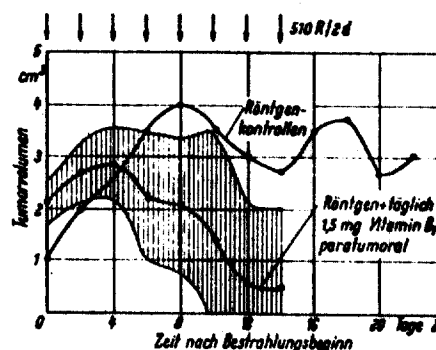


Abb. 4. Reaches Absterben der Tiere unter parenteraler Vitamin-B₁-Verabreichung und Röntgenbestrahlung gegenüber Röntgenkontrollen.

Abb. 5. Signifikante Differenz in der Tumordimensionentwicklung unter Röntgenbestrahlung und täglicher paratumoraler Verabreichung von Vitamin B₁ (schraffierte Fläche = 3mal mittlerer Fehler des Mittelwertes).



Bei direkter Einwirkung des Thiamins am Tumorbett war wohl ein gegenüber den Kontrollen signifikanter Unterschied in der Tumordimensionentwicklung erkennbar, jedoch war der Allgemeinzustand der Tiere infolge häufigen Auftretens von Nekrosen viel schlechter, was sich in den Absterbekurven widerspiegelt (Abb. 5).

Die Ergebnisse dieser ersten Untersuchungsreihe über die Verabreichung von Vitamin B₁ bei der Tumorbettbestrahlung lassen den Schluß zu, daß Thiamin wohl auf Grund seiner engen Beziehungen zum Kohlenhydratstoffwechsel eine Verbesserung der Sauerstoffutilisation einerseits im paratumoralen Gewebe, andererseits aber auch in der Tumorzelle selbst bewirkt. Was die Beeinflussung der Überlebensrate durch Vitamin B₁ bei Röntgenstrahleneinwirkung anbelangt, so sind auch die Ergebnisse anderer Untersuchungen, wie zum Beispiel die von Pfab [15] geprüfte Überlebenszeit bei der Ganzkörperbestrahlung von Kaulquappen, die einem Vitamin-B₁-haltigen Milieu ausgesetzt waren, und die Befunde von Rährlich [16], der ebenfalls Vitamin B₁ bei der Röntgenstrahlenwirkung an Kaulquappen erprobte, wenig überzeugend. Unsere Erfahrungen besagen, daß sich beim rasch entwickelnden Crocker-Sarkom an Mäusen des Inzuchtstammes NMRI unter dem Einfluß von Thiamin die Überlebenszeit der Tiere nicht verlängert, sondern im Gegenteil überraschenderweise verkürzt wird. Wir halten es daher für notwendig, auch an heterozygoten Mäusestämmen unter gleichen Versuchsbedingungen den Einfluß von Vitamin B₁ auf die Überlebenszeit der Tiere und das Tumordimension zu untersuchen, wie überhaupt weitere ähnliche Untersuchungen unter Verwendung anderer experimenteller Tumoren durchzuführen.

Zusammenfassung

An 235 Mäusen des homozygoten Stammes NMRI mit schnell wachsenden Tumoren (Crocker-Sarkom) vorgenommene Untersuchungen mit zusätzlicher paratumoraler Vitamin-B₁-Verabreichung zur fraktionierten Röntgenbestrahlung erbrachten signifikant bessere Volumendimensionen. Die Überlebenszeit der Tiere konnte jedoch unter dem Einfluß von Vitamin B₁ nicht verlängert werden. Es wird die Frage aufgeworfen, ob derartigen Untersuchungen an Inzucht-Tierstämmen nicht der generelle Nachteil anhaftet, daß solche Tiere a priori eine im Vergleich zu heterogenem Tiermaterial herabgesetzte Strahlenresistenz haben, was die Bewertung und Deutung der Ergebnisse erschwert.

Summary

Investigations concerning paratumoral application of vitamin B₁ in addition to fractionated X-ray irradiation showed a significantly better tumor regression. 235 mice of homozygous strain NMRI with rapidly growing tumors (Crocker sarcoma) were used. The survival time of the animals could not be prolonged by adding vitamin B₁. The question rises as to whether such investigations done with inbred animal strains do not have the general disadvantage, that these animals have a priori a decreased radiation resistance in comparison to heterogenous animal material. This makes evaluation and interpretation of the results more difficult.

Résumé

Des études sont faites sur 235 souris de souche homozygote NMRI porteuses de tumeurs à croissance rapide (sarcome de Crocker) avec administration paratumorale de vitamine B₁ complémentaire à une irradiation fractionnée. Ces expériences montrent une régression du volume tumoral nettement supérieure, toutefois sans augmentation de survie. Les auteurs se demandent si, dans de telles expériences, le fait d'utiliser des animaux de souche pure, dont la moindre radiorésistance est bien connue, ne constitue pas un inconvénient. Ceci rendrait plus difficile l'interprétation des résultats.

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EFFECT OF HIGH THIAMINE DOSES ON THE ECG OF THE RABBIT

by

J. R. Boissier, P. Simon, S. Witchitz and P. Viars

The use in anesthesiology of high i.v. doses of thiamine was recently recommended by De Castro (3). As a result of the noticeable harmlessness of the method mentioned by this author, we approached the problem of the toxicity of thiamine administered i.v. in the artificially ventilated animal and showed that the rabbit under these conditions, withstood the i.v. injection of doses about ten times higher than the non-ventilated rabbit (1, 2). Because of the negative inotropic and chronotropic actions of thiamine (5, 6), it seemed interesting to us to study the EKG changes induced by the i.v. administration in the rabbit of massive doses of this vitamin, either by acute injection or by perfusion.

In order to make allowance for changes due to the acidity of the solution, we compared the effects of the perfusion of the thiamine solution and of solutions neutralized by monosodium carbonate or THAM.

In order to put ourselves in the same conditions as De Castro, we studied the effects of thiamine put in solution in isotonic glucose solution.

METHODS

The male rabbits weighing from 2000 to 3400 g were tracheotomized and kept under artificial respiration by means of a Palmer pump. They then received:

--either an i.v. injection of a solution of thiamine hydrochloride at 160 g/l. The total volume of solution (corresponding to doses of thiamine between 400 and 1000 mg per kg of weight) was injected in 30 seconds into a marginal vein of the ear.

The profile was registered before the injection then every five minutes.

--or a continuous perfusion at the rhythm of 2 ml per minute of one of the following solutions prepared extemporaneously (six rabbits per batch):

- a. Thiamine hydrochloride 10 g
- qsp distilled water 100 ml (pH = 2.85)

The other problems of rhythm (fibrillation or idioventricular rhythm) have totally regressed in two cases and 30 minutes after the injection, the ECG had retaken its initial appearance (Fig. 1). In the seven other cases, the final evolution was heart failure.

The reappearance in six cases of the P waves appeared to us particularly noticeable; it evolved two times toward a definitive sinus rhythm with survival, two times toward a temporary sinus rhythm preceding heart failure and two times toward a complete auricular ventricular dissociation (4/1 and 2/1) also prefatal.

The rapid complex reappeared within the 15 minutes which followed the injection either in the form of an idioventricular rhythm (four cases), or in the form of a QRS complex at first appearance normal.

On the whole, the ventricular fibrillation has evolved:

- toward immediate death (two cases),
- toward an idioventricular rhythm followed by death (four times),
- toward a definitive normalization of the profile (two times).

2. Perfusion.

The fatal doses for each animal are indicated in Table 1. The evolution of the cardiac rhythm is indicated in Figures 2 and 3.

a. Thiamine solution.

An appreciable bradycardia has appeared for thiamine doses in the neighborhood of 4 g/kg. In addition we observed:

- a progressive and precocious disappearance of the P waves (five cases),
- changes of the rapid complex; enlargement (two cases), micro-voltage (three cases), axial rotation (two cases),
- repolarization problems: flat T (two cases), ST sub-contouring (four cases).

In one of the rabbits, the only change observed was a bradycardia increasing until heart failure.

b. A solution of thiamine + glucose.

If the presence of glucose does not bring changes to the evolution of the bradycardia, it however seems to be responsible for several changes:

- the P wave has never been changed in its morphology,
- the QRS voltage constantly increases without other change of the rapid complex than a widening (one case) and a right axial rotation (one case),
- the T wave is still increased in its voltage and tends to become symmetric,
- ST segment remains scarcely changed.

On the other hand, in one of the rabbits monomorphic extrasystoles appeared.

c. Thiamine solution + CO_3HNa .

The appearance of the bradycardia was retarded (Fig. 3).

In addition we observed:

- in all cases, problems of repolarization causing a very considerable hypokalemia and appearing for doses between four and eight g/kg,
- three times an appearance of the P wave,
- three times an auriculo-ventricular dissociation, the QRS voltage increasing before the terminal disappearance,
- two times a left axial rotation.

d. Thiamine solution + THAM.

The presence of THAM did not change the appearance speed of the bradycardia. We observed:

- in all cases, the same problems of repolarization as in the presence of CO_3HNa ,
- one time the disappearance of the P wave,
- two times an axial rotation (one right and one left).

DISCUSSION

Thiamine has a very weak cardiac toxicity.

The acute injection administration brings problems only for doses equal to or superior to 800 mg/kg. Most often it is a question of ventricular fibrillation of which the spontaneous and definitive reversibility in two cases is quite noticeable.

Under venous perfusion, the appearance of the bradycardia is constant and the intensity of this bradycardia is grossly proportional to the dose. Whatever the solution used, no major problem appeared for doses of thiamine less than 4 g/kg. Changes of the ventricular complex and of the repolarization are scarcely evident and consist mainly in ECG signs such as they are encountered in the states of hypokalemia, when the solution is neutralized by the THAM or the CO_3HNa . The effacement then the frequent disappearance of the P wave seem to indicate a change of the sinus all the more accentuated as the dose of thiamine is raised.

However, in the presence of glucose, or reasons perhaps metabolic, the absence of change of the P wave translates a conservation of the activity of the sinus.

On a practical plane the ECG changes induced by thiamine administered in perfusion in the rabbit appear only for very high doses, much greater than those used in anesthesiology. These facts are in agreement with the absence of significant changes of the ECG in subjects having received in an "anesthetic" goal high doses of thiamine in venous perfusion (4).

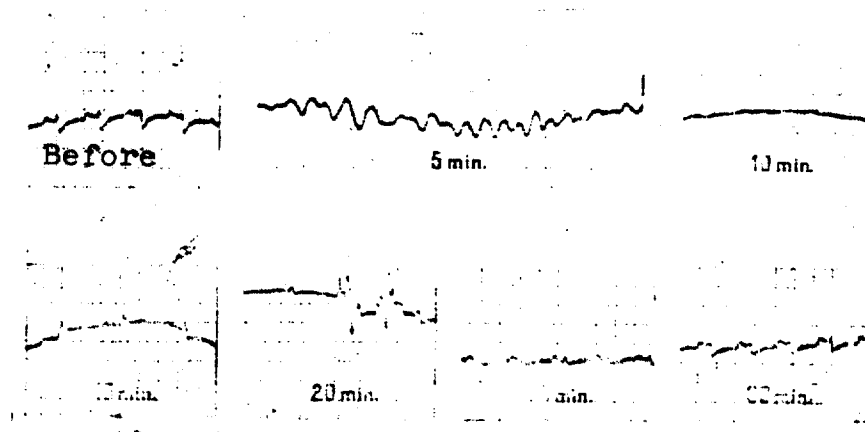


Fig. 1. -- Male rabbit -- 2100 g. Artificial respiration, ECG (D_3 derivation). I.V. injection in 30 seconds of thiamine hydrochloride: 900 mg/kg (solution at 160 g/l).

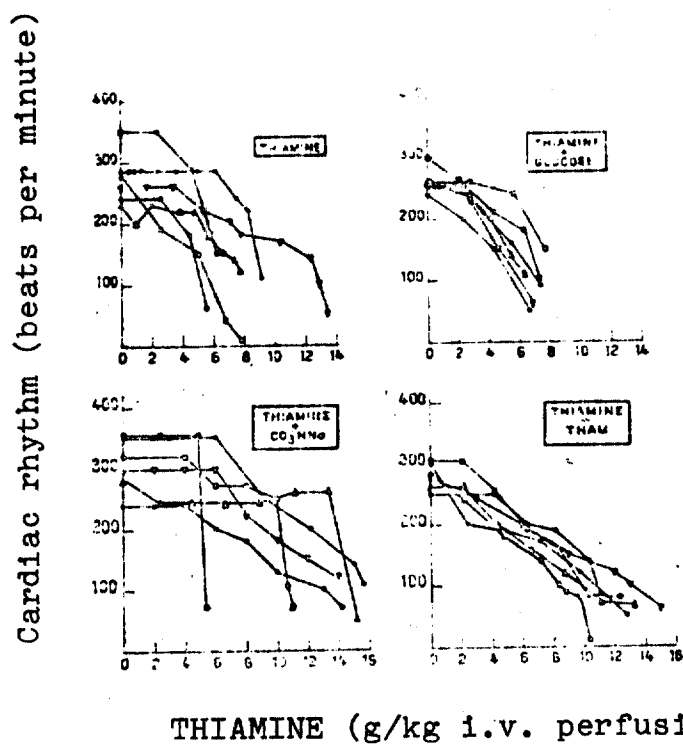


Fig. 2. -- Evolution of the cardiac rhythm under the influence of the perfusions of thiamine solution in the rabbit: each curve represents one animal.

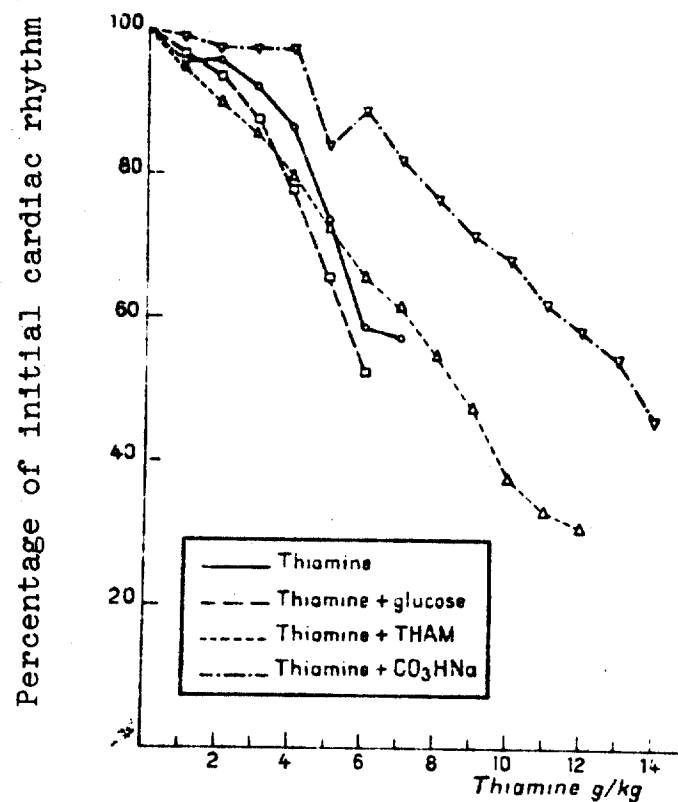


Fig. 3 -- The evolution of the cardiac rhythm under the influence of the perfusions of thiamine solution in the rabbit: each curve represents the average of the batch of animals. The average percentages were calculated only when there were at least three rabbits per batch.

Table 1.

Perfused Solution	Lethal Doses of Thiamine (in g/kg)	Average
Thiamine	5.45; 6.15; 7.23; 9.16; 9.20; 13.52	8.47
Thiamine + glucose	6.30; 6.66; 6.92; 7.14; 7.50; 7.92	7.03
Thiamine + CO ₂ HNa	5.20; 11.73; 14.0; 14.60; 15.55; 16.20	12.86
Thiamine + THAM	10.40; 11.63; 12.41; 12.80; 13.33; 21.0	14.19

Anesth., Analg., Reanim. 23(3):557-564. 1966.

Anesth. Anal. Réan., 1966, 23, 3.

XV^e CONGRÈS FRANÇAIS D'ANESTHÉSIE-RÉANIMATION

ACTION DES FORTES DOSES DE THIAMINE SUR L'ECG DU LAPIN (*)

PAR

J.-R. BOISSIER (**), R. SIMON, S. WITCHITZ et P. VIARS

(Paris)

L'utilisation en anesthésiologie de fortes doses de thiamine par voie veineuse a été préconisée récemment par DE CASTRO (3). A la suite de la remarquable innocuité de la méthode signalée par cet auteur, nous avons abordé le problème de la toxicité de la thiamine administrée par voie veineuse chez l'Animal artificiellement ventilé et montré que le Lapin supportait dans ces conditions l'injection intraveineuse de doses environ dix fois plus fortes que le Lapin non ventilé (1, 2). En raison des actions inotrope et chronotrope négatives de la thiamine (5, 6), il nous a paru intéressant d'étudier les modifications électrocardiographiques induites par l'administration veineuse chez le Lapin de doses massives de cette vitamine, soit en injection aiguë, soit en perfusion.

Afin de tenter de faire la part des modifications dues à l'acidité de la solution, nous avons comparé les effets de la perfusion de la solution de thiamine et de solutions neutralisées par le carbonate monosodique ou le THAM.

Enfin, pour nous placer dans les mêmes conditions que DE CASTRO, nous

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avons étudié les effets de la thiamine mise en solution dans du soluté glucosé isotonique.

METHODES

Les lapins mâles pesant de 2 000 à 3 400 g étaient trachéotomisés et maintenus sous respiration artificielle à l'aide d'une pompe de Palmer. Ils recevaient alors :

— soit une injection intraveineuse d'une solution de chlorhydrate de thiamine à 160 g/l. Le volume total de solution (correspondant à des doses de thiamine comprises entre 400 et 1 000 mg par kilogramme de poids) était injecté en 30 secondes dans une veine marginale de l'oreille.

Le tracé ECG était enregistré avant l'injection puis toutes les 5 minutes.

— soit une perfusion continue au rythme de 2 ml par minute d'une des solutions suivantes préparées extemporanément (6 lapins par lot) :

a. Chlorhydrate de thiamine	10 g
Eau distillée q.s.p.	100 ml (pH = 2,85)
b. Chlorhydrate de thiamine	10 g
Carbonate monosodique	4,5 g
Eau distillée q.s.p.	100 ml (pH = 7,1)
c. Chlorhydrate de thiamine	10 g
THAM	4,5 g
Eau distillée q.s.p.	100 ml (pH = 7,4)
d. Chlorhydrate de thiamine	10 g
Soluté isotonique de glucose q.s.p.	100 ml (pH = 3).

Le tracé ECG était enregistré avant le début de la perfusion puis toutes les 15 minutes jusqu'à la mort de l'animal.

Toutes les doses sont exprimées en chlorhydrate de thiamine.

RÉSULTATS

1. Injection rapide.

Le DL₅₀ dans ces conditions expérimentales est voisine de 550 mg/kg, aucun animal ne survécut après 1 000 mg/kg. Cette donnée est en accord avec les résultats que nous avions trouvés antérieurement (1, 2).

L'injection de doses de thiamine inférieures à 800 mg/kg n'a pas entraîné de modifications notables de l'ECG : seule une sous-dépression discrète et transitoire du segment ST est apparue deux fois sur quatre ; tous les animaux ont survécu.

Des altérations importantes du tracé ECG apparaissent après l'injection de doses de thiamine comprises entre 800 et 1 000 mg/kg (15 lapins). Trois lapins ayant reçu 800 mg/kg ont gardé un tracé ECG normal.

Nous avons observé :

- trois fois une bradycardie sinusale,
- une fois l'apparition d'un rythme idioventriculaire (dans ces quatre cas, le voltage du complexe rapide avait notablement diminué, l'onde P étant diminuée ou disparue ; dans deux cas on observait une sous-dépression du segment ST),
- huit fois une fibrillation ventriculaire.

La surveillance du tracé ECG a montré l'évolution suivante :

La bradycardie sinusale a toujours régressé en moins de 30 minutes, avec normalisation du tracé.

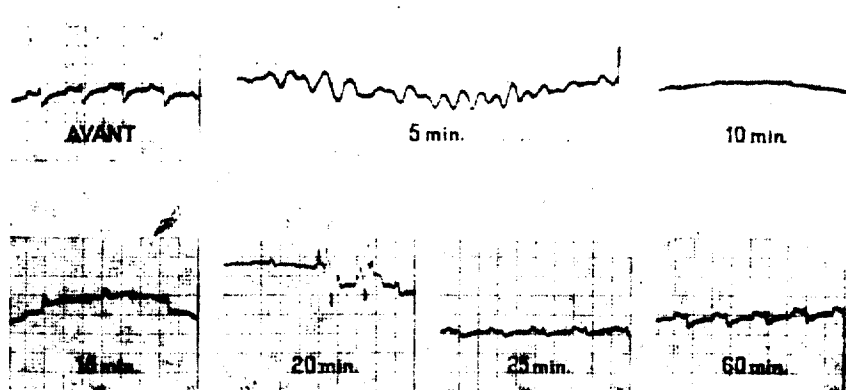


FIG. 1. — Lapin mâle — 2 100 g. Respiration artificielle. E. C. G. (dérivation D₃).
Injection intraveineuse en 30 secondes de chlorhydrate de thiamine : 1000 mg/kg (solution à 100 g/l).

Les autres troubles du rythme (fibrillation ou rythme idioventriculaire) ont dans deux cas totalement régressé et 30 minutes après l'injection, l'ECG avait repris son aspect initial (fig. 1). Dans les sept autres cas, l'évolution finale a été l'arrêt cardiaque.

La réapparition dans six cas des ondes P nous a paru particulièrement remarquable ; elle a évolué deux fois vers un rythme sinusal définitif avec survie, deux fois vers un rythme sinusal temporaire précédant l'arrêt cardiaque et deux fois

vers une dissociation auriculoventriculaire complète (4/1 et 2/1) également pré-mortelle.

Le complexe rapide réapparaissait dans les 15 minutes qui suivaient l'injection soit sous forme d'un rythme idioventriculaire (quatre cas), soit sous forme d'un complexe QRS d'emblée normal.

Au total, la fibrillation ventriculaire a évolué :

- vers la mort immédiate (deux fois),
- vers un rythme idioventriculaire suivi de mort (quatre fois),
- vers une normalisation définitive du tracé (deux fois).

2. Perfusion.

Les doses mortelles pour chaque animal sont indiquées sur le tableau I. L'évolution du rythme cardiaque est indiquée sur les figures 2 et 3.

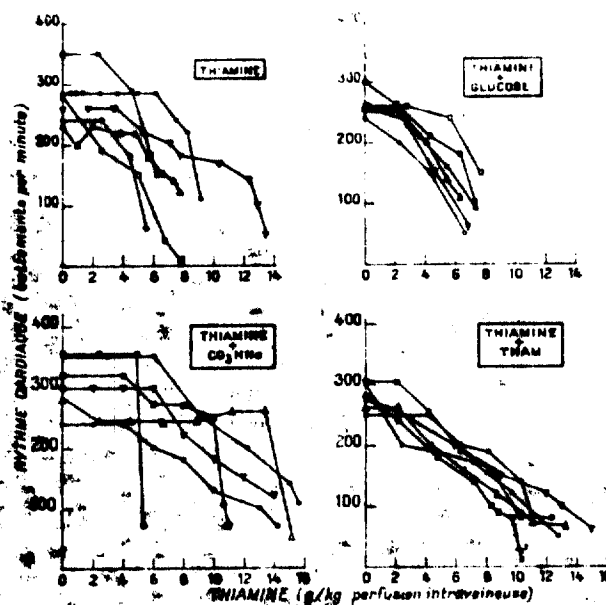


Fig. 2. — Evolution du rythme cardiaque sous l'influence des perfusions de solution de thiamine chez le lapin : chaque courbe représente un animal.

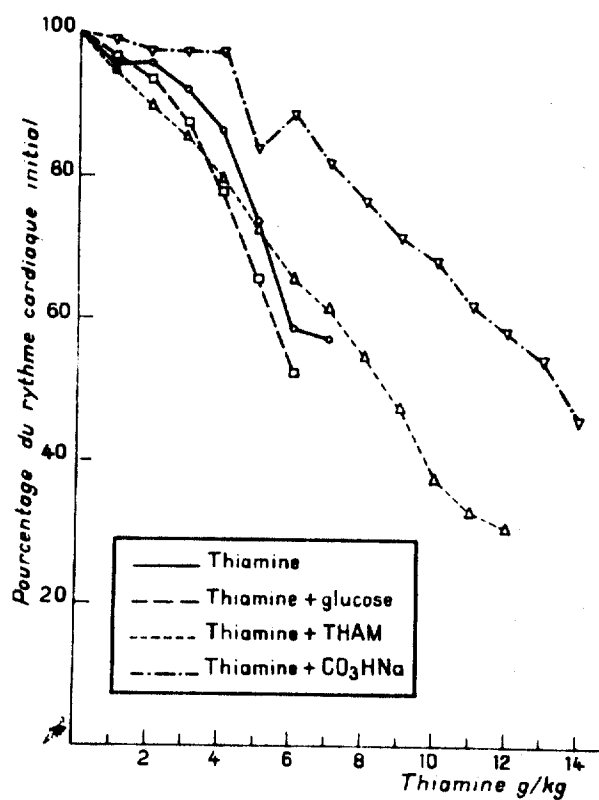


Fig. 3. -- Évolution du rythme cardiaque sous l'influence des perfusions de solution de thiamine chez le Lapin : chaque courbe représente la moyenne du lot d'animaux. Les pourcentages moyens n'ont été calculés que lorsqu'il y avait au moins encore 5 Lapins par lot.

TABLEAU I

Solution perfusée	Doses mortelles de thiamine (en g/kg)	Moyenne
Thiamine	5,45 ; 6,15 ; 7,25 ; 9,16 ; 9,27 ; 13,52	6,47
Thiamine + glucose	6,30 ; 6,60 ; 6,62 ; 7,14 ; 7,50 ; 7,62	7,03
Thiamine + CO ₃ HNa	5,20 ; 11,73 ; 14,0 ; 14,66 ; 15,55 ; 16,20	12,86
Thiamine + THAM	10,40 ; 11,03 ; 12,41 ; 12,80 ; 13,33 ; 21,0	14,19

a. Solution de thiamine.

Une bradycardie notable est apparue pour des doses de thiamine voisines de 4 g/kg. Nous avons en outre observé :

- une disparition progressive et précoce des ondes P (cinq cas),
- des modifications du complexe rapide : élargissement (deux cas), micro-voltage (trois cas), rotation axiale (deux cas),
- des troubles de la repolarisation : T plat (deux cas) sous dénivellation de ST (quatre cas).

Chez un des lapins, la seule modification observée a été une bradycardie s'accroissant jusqu'à l'arrêt cardiaque.

b. Solution de thiamine + glucose

Si la présence de glucose n'apporte pas de modification à l'évolution de la bradycardie, elle semble cependant être responsable de quelques modifications :

- l'onde P n'a jamais été altérée dans sa morphologie.
- le voltage de QRS augmente constamment sans autre modification du complexe rapide qu'un élargissement (un cas) et une rotation axiale droite (un cas),
- l'onde T est toujours augmentée dans son voltage et tend à devenir symétrique.
- le segment ST reste peu modifié.

Par ailleurs, chez un des lapins sont apparues des extrasystoles monomorphes.

c. Solution de thiamine + CO_2HNa .

La bradycardie a été retardée dans son apparition (fig. 3).

Nous avons en outre observé :

- dans tous les cas des troubles de la repolarisation évoquant une hypokaliémie très importante et apparaissant pour des doses situées entre 4 et 8 g/kg,
- trois fois une disparition de l'onde P,
- trois fois une dissociation auriculo-ventriculaire, le voltage de QRS augmentant avant la disparition terminale,
- deux fois une rotation axiale gauche.

d. Solution de thiamine + THAM.

La présence de THAM n'a pas modifié la vitesse d'apparition de la bradycardie. Nous avons observé :

- dans tous les cas, les mêmes troubles de la repolarisation qu'en présence de CO_2HNa ,
- une fois la disparition de l'onde P,
- deux fois une rotation axiale (une droite et une gauche).

DISCUSSION

La thiamine possède une toxicité cardiaque très faible.

L'administration en injection aiguë n'entraîne de troubles que pour les doses égales ou supérieures à 800 mg/kg. Il s'agit le plus souvent de fibrillation ventriculaire dont la réversibilité spontanée et définitive dans deux cas est tout à fait remarquable.

Sous perfusion veineuse, l'apparition de la bradycardie est constante et l'intensité de cette bradycardie est grossièrement proportionnelle à la dose. Quelle qu'ait été la solution employée, aucun trouble majeur n'est apparu pour les doses de thiamine inférieures à 4 g/kg. Les modifications du complexe ventriculaire et de la repolarisation sont peu marquées et consistent surtout en signes ECG tels qu'on les rencontre dans les états d'hypokaliémie, lorsque la solution est neutralisée par le THAM ou le CO_2HNa . L'effacement puis la disparition fréquente de l'onde P semblent témoigner d'une altération du sinus d'autant plus accentuée que la dose de thiamine est plus élevée.

Cependant, en présence de glucose, pour des raisons peut être métaboliques, l'absence de modification de l'onde P traduit une conservation de l'activité du sinus.

Sur un plan pratique les modifications ECG induites par la thiamine administrée en perfusion chez le Lapin n'apparaissent que pour des doses très élevées, très supérieures à celles utilisées en anesthésiologie. Ces données sont en accord avec l'absence de modifications significatives de l'ECG chez les sujets ayant reçu dans un but « anesthésique » de fortes doses de thiamine en perfusion veineuse (4).

RÉSUMÉ

Les auteurs ont analysé les modifications électrocardiographiques induites dans des conditions expérimentales différentes sous l'influence de doses massives de thiamine chez le Lapin artificiellement ventilé :

— l'injection veineuse brutale n'entraîne de modifications qu'aux doses voisines des doses mortelles ; il s'agit le plus souvent d'une fibrillation ventriculaire, parfois réversible spontanément.

— la perfusion lente entraîne une bradycardie proportionnelle à la dose. Les modifications de l'aspect des tracés provoquées par la neutralisation de la solution (THAM ou CO_2HNa) ou par l'adjonction de glucose sont discutées.

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ORIGINAL ARTICLES

OBSERVATIONS ON THE ROLE OF VITAMIN B₁ IN THE ETIOLOGY AND TREATMENT OF KORSAKOFF PSYCHOSIS*

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The possibility that some of the mental disorders occurring in the alcohol addict may be due to vitamin deficiency has gained increasing attention since the recognition that vitamin undersaturation (1) and clinical vitamin deficiency (2) is common in alcohol addicts. Vitamin B₁ deficiency, as manifested by polyneuritis, was found by Romano (3) in 58 per cent of his patients having alcoholic psychoses. Jolliffe and Frank (4) found polyneuritic signs in 61.6 per cent of 1,000 consecutive male alcoholic admissions to the Psychiatric Division of Bellevue Hospital. Applying the minimum criteria of Goodhart and Jolliffe (5) for the diagnosis of polyneuritis, about one-third of Romano's patients, and 22.6 per cent of our material had definite polyneuritis.

Vitamin B₁ deficiency has been considered by both Strauss (6) and Weiss (7) as a factor responsible for the psychosis in Korsakoff syndrome. Strauss states that "... the Korsakoff syndrome and other similarly misleading names have concealed the true diagnosis of vitamin B₁ deficiency in the western world." Weiss states that "it is possible that the etiology of this condition, just as in alcoholic polyneuritis,

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nutritional deficiency, particularly chronic deficiency of vitamin B₁, plays a role." This hypothesis is supported by the high incidence of polyneuritis in patients with Korsakoff psychosis observed in alcohol addiction, pregnancy, hyperthyroidism, and diabetes mellitus. The characteristic polyneuritis in these conditions is considered to be due to vitamin B₁ deficiency, and responds, if not advanced to irreversible pathologic changes, to adequate thiamin therapy. It is therefore not illogical to postulate that both the psychosis and the polyneuritis have the same etiological basis. The Korsakoff psychosis, however, also occurs in conditions not associated with vitamin undersaturation or clinical vitamin deficiency, as head trauma and asphyxia.

The purpose of the present report is to present the evidence available from our (2, 5, 8, 9, 10) studies of patients having polyneuritis, concerning the possible role of vitamin B₁ deficiency in the production of Korsakoff psychosis, and to evaluate the usefulness of thiamin (crystalline vitamin B₁) therapy in the treatment of patients having this mental picture.

METHODS

The criteria used for the diagnosis of Korsakoff psychosis required the presence of a triad consisting of gross memory defects for recent events, disorientation, and confabulation. We are cognizant of the fact that many neuro-psychiatrists do not make a diagnosis of Korsakoff psychosis unless this triad of mental symptoms persists for a month or longer. We have not made this distinction in this study. The subjects (31) who at the onset or during the course of the psychosis showed, in addition to this triad, delirium, encephalopathy, acute hallucinosis, or a combination of these, are designated as having Korsakoff psychosis with acute onset. Those (20) who presented only the classical triad, without these added manifestations, are designated as having Korsakoff psychosis with insidious onset.

The 51 subjects with Korsakoff psychosis included in this study were observed on the Medical Service of the Psychiatric Division of Bellevue Hospital during the two years ending March 31, 1938. These patients were admitted to and studied on the medical service because of polyneuritis or other somatic disease; and the therapy in each subject was determined by a system of rotation used in evaluating thiamin as a therapeutic agent in polyneuritis (5). Upon admission to the medical service, all 51 subjects were maintained with our basal diet (9) for a period of 1 to 30 (average 11) days. This diet supplied a vitamin B₁/calory ratio (11) of 1.7, which is of borderline adequacy in vitamin B₁ for a subject weighing 60 kg. As Jolliffe and Colbert (9) have

previously shown, subjects with polyncuritis due to vitamin B₁ deficiency will not show improvement in objective signs of peripheral neuritis even if maintained with this diet for as long as 90 days.

During the period of basal diet observations, 15 subjects were changed. Those remaining (36 subjects) were subsequently given a weighed vitamin B₁ rich diet (9) plus 18 grams of vegex (12) daily. This regimen was estimated to supply 1,066 international units of vitamin B₁ (about 33 mg. of thiamin) and a vitamin B₁/calory ratio of 0.8, which supplies approximately 4 times the predicted vitamin B₁ requirement (11) of a subject weighing 60 kg. The 15 subjects who received this and no further treatment are designated as group C. The remaining 21 subjects were subsequently treated with 10 to 50 mg. of thiamin (13) daily by parenteral injection, in addition to the weighed vitamin B₁ rich diet and vegex. These are designated as group D. Of the 21 subjects in group D, 10 received 10 mg. of thiamin daily, and 11 received 20 mg. or more daily. The treatment received by patients in group C and group D is identical with the treatment received by the corresponding groups in the previous studies of Jolliffe and Colbert (9) and of Goodhart and Jolliffe. (5)

We have not attempted to grade improvement in the mental picture, other than to record while under observation the occurrence or failure of recovery from the psychosis. The disappearance of confabulation and disorientation has been taken as the criterion of recovery. Memory defects of varying degree remained in nearly every case, whether the onset of the psychosis was insidious or with acute manifestations, and irrespective of the treatment given.

RESULTS

Recovery from the Korsakoff psychosis occurred in 17 (33.3%) of the 51 patients in this study. The average period of observation prior to recovery was 24 days, varying from 6 to 53 days. The average period of observation of the patients who did not recover was 31 days, varying from 6 to 127 days. Recoveries occurred most commonly in the 3rd and 4th week, as illustrated by the following tabulations: 1st week, 2; 2nd week, 3; 3rd week, 4; 4th week, 3; 5th week, 2; 6th week, 2; after 6th week, 2.

The relationship of type of treatment to recovery from the Korsakoff psychosis is summarized in table 1. The 51 subjects were first maintained with the basal diet for 1 to 30 (average 11) days. Recovery from the Korsakoff psychosis occurred in 6 subjects (11.8%) during the basal diet period.

TABLE 1.—THE RELATIONSHIP OF TYPE OF TREATMENT TO RECOVERY FROM THE KORSAKOFF PSYCHOSIS

Subjects in study.....	51
Maintained with basal diet (Average 11 days).....	51
Recovered.....	6 (11.8%)
Discharged unimproved.....	9
Remaining for observation.....	36
Treated in Group C (average 18 days).....	15
Recovered.....	1 (6.6%)
Discharged unimproved.....	14
Treated in Group D (average 29 days).....	21
Recovered.....	10 (47.6%)
Discharged unimproved.....	11

As 9 additional subjects were discharged, unimproved, 36 remained for further observation. Of these, 15 were treated in group C and 21 in group D. The patients in group C were maintained with the regimen described for 5 to 37 (average 18) days. Recovery from the Korsakoff psychosis occurred in 1 subject (6.6%) during this period. The patients in group D were maintained with the regimen described for 9 to 79 (average 29) days. Recovery from the Korsakoff psychosis occurred in 10 subjects (47.6%) during this period. The average period of treatment for subjects in group D was 11 days longer than for subjects in group C (29 and 18 days, respectively). The total period of observation averaged 28 days for the patients in group C and 38 days for those in group D, so that the latter had an average of 10 days more during which recovery might occur spontaneously.

A comparison of the 31 subjects having an acute onset with the 20 subjects having an insidious onset is summarized in table 2. Re-

TABLE 2.—THE RELATIONSHIP OF TYPE OF TREATMENT TO RECOVERY FROM THE KORSAKOFF PSYCHOSIS WITH AN "ACUTE" OR WITH AN "INSIDIOUS" ONSET

	Onset		Total
	Acute	Insidious	
Subject maintained with basal diet.....	31	20	51
Recovered.....	5 (16.1%)	1 (5.0%)	6 (11.8%)
Discharged unimproved.....	2	7	9
Remaining for further observation.....	24	12	36
Treated in Group C.....	8	7	15
Recovered.....	1 (12.5%)	0	1 (6.6%)
Discharged unimproved.....	8	7	15
Treated in Group D.....	16	5	21
Recovered.....	9 (56.2%)	1 (20.0%)	10 (47.6%)
Discharged unimproved.....	7	4	11

recovery from the Korsakoff psychosis while under observation occurred in 15 (48.4%) of the 31 patients whose psychosis began acutely. These subjects were first maintained with the basal diet for 1 to 27 (average 10) days. During this period recovery occurred in 5 subjects (16.1%). These 5 and 2 additional subjects who had not improved were discharged without further treatment, leaving 24 subjects for further study. Of these, 8 were treated in group C and 16 in group D. The 8 subjects in group C were treated for 5 to 17 (average 11) days. Recovery was noted in 1 (12.5%). The 16 subjects in group D were treated for 9 to 39 (average 24) days. Recovery was noted to occur in 9 (56.2%). The total period of observation averaged 18 days for the patients in group C and 33 days for those in group D, so that the latter had an average of 15 days more during which recovery might occur.

The 20 subjects having an insidious onset were observed for 6 to 127 (average 35) days. Recovery from the Korsakoff psychosis was noted to occur in 2 (10%) of these subjects. These subjects were first maintained with the basal diet for 3 to 30 (average 13) days. During this period 1 (5%) recovered from the psychosis (on the 16th day) and 7 were discharged unimproved, leaving 12 subjects for further study. Of these, 7 were treated in group C and 5 in group D. Recovery was not noted in any of the 7 subjects in group C, although they were treated for an average of 28 (10 to 97) days. Recovery was noted in 1 (20%) of the 5 subjects in group D, who were treated for an average of 48 (15 to 70) days. Because of the small number of cases in these subgroups, the occurrence of 1 recovery in group D and of none in group C does not lend itself to interpretation.

DISCUSSION

The above findings indicate the difficulty of drawing conclusions as to either etiology or effectiveness of treatment from the results of specific therapy in a disease with so varied an onset and course as that of Korsakoff psychosis. In this disease, when the onset is acute, that is associated with delirium, acute hallucinosis or encephalopathic manifestations, the immediate prognosis is serious, but if such patients survive intercurrent infection, circulatory collapse or encephalopathy, the prognosis for recovery from the Korsakoff psychosis is excellent, in that about 50 per cent recover within one month. We have, however, found no criteria by which we can predict this early recovery. When the disease has an insidious onset, the prognosis for survival is excellent; but the psychosis is more chronic in course, and recovery within a month occurs in only about 10 per cent of the subjects. In view of

this, any reports of recovery from Korsakoff psychosis as due to specific therapy must be examined critically.

In the group as a whole, when maintained with a diet of borderline adequacy in vitamin B₁, recovery was noted in about 12 per cent of the subjects during an average of 11 days of observation. On this regimen, not one of these subjects demonstrated any improvement in the objective signs of polyneuritis. This observation does not support the hypothesis that Korsakoff psychosis is a manifestation of vitamin B₁ deficiency. The higher incidence of recovery in those treated with thiamin plus a vitamin rich diet (group D) than those treated by the same vitamin rich diet alone (group C) does lend support to the hypothesis that Korsakoff psychosis is a manifestation of vitamin B₁ deficiency. It seems to us that the increased incidence of recovery in group D over group C must be attributed either to the thiamin received by group D, or to spontaneous recovery not related to treatment. In an attempt to rule out spontaneous recovery as a factor in the apparently better results in the group treated with thiamin, we are confronted with the fact that when group C and D are divided into subgroups according to the type of onset, too few subjects, particularly in the group with an insidious onset, remain for statistical analysis. It is suggestively significant, however, that in our subjects the incidence of recovery from the Korsakoff psychosis was 7 times greater in subjects treated with added thiamin than in those not so treated. It seems to us that this problem should be studied further by the same or similar methods, in a larger group of patients and over a longer period of time, before any attempt can be made to draw definite conclusions as to whether the Korsakoff psychosis is a manifestation of vitamin B₁ deficiency and whether thiamin has specific value in the therapy of the psychosis.

SUMMARY AND CONCLUSIONS

We have studied 51 subjects having Korsakoff psychosis who were maintained first with a diet of borderline adequacy in vitamin B₁ for an average of 11 days during which time 11.7 per cent recovered. On conclusion of this control period, 36 patients were given a weighed vitamin rich diet which, with the added vegex, was estimated to contain 1,066 international units of vitamin B₁ (3.3 mg. thiamin). This diet supplied a vitamin B₁/calory ratio of approximately 4 times the minimal predicted requirement of vitamin B₁ for a subject weighing 60 kg. Twenty of these subjects received in addition, 10 to 50 mg. of thiamin daily by parenteral administration. Subjects receiving thiamin showed an incidence of recovery approximately 7 times as great as those

treated by the diet alone. This conclusion, however, cannot be confirmed by statistical analysis, and we are unable definitely to state whether the increased incidence of recovery in the group receiving thiamin was due to the therapy or due to spontaneous recoveries not related to therapy.

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Effect of Dietary Level of Thiamine on Reproduction in the Rat

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ABSTRACT Reproductive performance and certain aspects of maternal biochemical response were studied in female rats of the Wistar strain maintained with a basal diet containing either an adequate or a low level of thiamine from weaning until mating. During pregnancy the rats were fed an adequate basal diet, or the same basal diet with all vitamin levels doubled. Fewer young were produced by animals fed at the low level of thiamine prior to pregnancy. Albumin and γ -globulin were the only serum proteins that appeared to be influenced by thiamine intake. In other respects reproductive performance and maternal biochemical response were normal even when feed intake and thiamine level were low prior to pregnancy. The young were of normal weight. Total serum protein, hemoglobin and hematocrit were similar. Doubling the intake of thiamine and other vitamins during pregnancy had no effect on reproductive performance. Thiamine content in the young and in livers of mothers was related to thiamine intake during pregnancy and was not influenced by low thiamine level prior to pregnancy.

Although the effect of the total absence of dietary thiamine upon reproductive performance of the rat has been investigated (1, 2), the effect of a prolonged partial deficiency has not been reported. The present study is concerned with the effect of a chronic thiamine deficiency from weaning until mating, and the reproductive performance and maternal biochemical response when thiamine levels during pregnancy meet or exceed those generally considered adequate.

EXPERIMENTAL

Female rats of the Wistar strain were fed ad libitum from weaning a basal diet containing initially either 2.5 or 0.5 mg thiamine/kg diet. The basal diet consisted of the following: (in per cent) vitamin-free casein, 25; sucrose, 40.75; cornstarch, 18; beef suet, 8; corn oil, 2; salts (3), 4; cellulose,² 2; L-cystine, 0.15; and choline chloride, 0.1. Other vitamins were added in the following amounts per kilogram of diet: (in milligrams) riboflavin, 5; Ca pantothenate, 25; niacin, 10; pyridoxine-HCl, 2.5; folic acid, 2; biotin, 0.2; vitamin B₁₂, (0.1% mannitol trituration), 0.2; inositol, 200; p-aminobenzoic acid, 100; and menadione, 2.5. Fat-soluble vitamins were administered once weekly in 0.05 ml of

corn oil and supplied 5 mg *dl*- α -tocopheryl acetate, 1250 IU vitamin A acetate and 12 IU vitamin D₃ per rat per week.

To permit normal estrous cycles, the thiamine level of the diet containing originally 0.5 mg/kg was raised progressively to 0.75 mg/kg after 4 weeks and to 1.0 mg/kg at the beginning of the sixth week of experiment.

At approximately 70 days of age, animals were mated with male Wistar rats that had been reared with commercial laboratory chow,³ and if sperm were observed in the vaginal smear, the rats were considered pregnant. Following mating, some of the animals of each initial group were fed the basal diet containing 2.5 mg thiamine/kg of diet. Others were fed the basal diet in which levels of all vitamins except choline were doubled. This diet hereafter will be designated as the 5.0 mg/kg diet. Animals were weighed and vaginal smears examined daily to detect any deviation from the normal course of pregnancy.

On the 22nd day of gestation, the animals were anesthetized with sodium amytal.

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² Cellu Flour, Chicago Dietetic Supply House, Chicago.

³ Purina Laboratory Chow, Ralston Purina Company, St. Louis.

the Blood was removed by tail bleeding for determination of hematocrit⁴ and hemoglobin⁵ and from the heart for determination of total serum protein (4) and serum protein fractions by paper electrophoresis.⁶ Maternal livers and entire litters were removed and frozen immediately and were analyzed later for thiamine using the thiochrome method (5).

RESULTS

Maternal weight gain and reproductive performance. Animals fed 2.5 mg thiamine/kg prior to pregnancy were approximately 65 g heavier at the time of mating than those fed 0.5 to 1.0 mg/kg. Total weight gain during pregnancy was similar in all groups (table 1). Rate of gain, however, differed significantly with level of thiamine fed prior to pregnancy. Animals fed at the lower level gained more during the first week of pregnancy ($P < 0.01$); animals fed at the higher level gained more during the last week ($P < 0.05$).

Animals reared with the basal ration produced larger litters than those fed at the lower level of thiamine prior to mating (table 1). Doubling the level of vitamins fed during pregnancy had no effect on litter size. Weight of young and number of resorptions were not significantly different among the diet groups.

Serum protein, hematocrit and hemoglobin. Total serum protein was similar in all groups (table 2). There was a consistent tendency for the percentage of albumin in the serum proteins to parallel the level of thiamine intake whether comparisons were based on pre-pregnancy or pregnancy levels, although the differences were not statistically significant. In con-

trast, γ -globulin was high when thiamine intake was low. The γ -globulin level of 7.1% observed in the sera of animals fed 0.5 mg/kg prior to pregnancy and 2.5 mg/kg during pregnancy was significantly higher than that of either group of animals fed 2.5 mg/kg prior to pregnancy ($P < 0.01$). An increase in thiamine intake from 0.5 mg/kg prior to pregnancy to 5.0 mg/kg during pregnancy resulted in a restriction in γ -globulin to a level similar to the higher pre-pregnancy levels of thiamine and differed significantly from the pregnancy level of 2.5 mg/kg ($P < 0.05$).

No differences in hemoglobin or hematocrit due to diet prior to or during pregnancy were observed.

Thiamine content of maternal livers and fetuses. The thiamine content of maternal livers and of the fetuses is shown in table 3. Significantly higher levels of thiamine ($P < 0.01$) were noted in livers and young of mothers fed 5.0 mg/kg during pregnancy than in those fed the diet containing 2.5 mg/kg. No differences due to diet prior to pregnancy were observed.

DISCUSSION

Thiamine deficiency is known to reduce fertility in the rat (1, 2). In the present study, the average number of resorptions was similar for all groups and was of the same order observed in animals fed stock diet and maintained under the same experimental conditions.⁷ The small size of

⁴ 73-mm capillary tubes centrifuged for 3 minutes at 12,300 rpm.

⁵ Hycel, Inc., Houston, Texas 1959 Cyanmethemoglobin Determination.

⁶ Beckman Instrument Company, Spinco Division, Model R Paper Electrophoresis Instruction Manual RIM-5, Palo Alto, California.

⁷ Unpublished data.

TABLE 1

Influence of level of dietary thiamine prior to and during pregnancy on weight gain and reproductive performance of the rat

Dietary thiamine		No. of litters	Avg maternal wt gain				Avg no. of young/litter	Avg fetal wt	Avg no. resorptions
Pre-pregnancy	Pregnancy		Week 1	Week 2	Week 3	Total			
mg/kg	mg/kg								
2.5	2.5	10	32	31	69	130	10.1	4.8	1.3
2.5	5.0	8	29	30	74	132	10.1	4.9	0.9
0.5-1.0	2.5	6	40	28	57	125	6.8	4.9	1.3
0.5-1.0	5.0	13	41	31	53	127	7.2	5.3	2.5

TABLE 2
Serum proteins, hematocrit, and hemoglobin of pregnant rats fed at different levels of thiamine prior to and during pregnancy

Dietary thiamine	Pre- pregnancy	Pregnancy	No. of sera analyzed	Serum proteins										Hematocrit	Hemoglobin
				Total	Albumin	Globulins									
						α_1	α_2	β	γ						
mg/kg	mg/kg	mg/kg		g/100 ml	%	%	%	%	%	%	%	%	%	g/100 ml	
2.5	2.5	2.5	9	5.7	39.5	29.1	11.2	15.4	5.1				35.5	12.2	
2.5	5.0	5.0	8	5.4	43.5	26.6	11.3	14.0	4.6				35.8	12.6	
0.5	2.5	2.5	6	6.0	36.0	27.3	12.0	17.6	7.1				36.0	11.8	
0.5	5.0	5.0	11	5.7	40.0	27.7	10.8	15.9	5.6				36.3	12.2	

litters in animals with a limited intake of thiamine prior to pregnancy therefore appears to reflect lowered fertility rather than a high resorption rate. The failure to affect litter size by increasing level of thiamine during pregnancy was further indication that litter size was the result of nutritional status at the time of conception rather than of diet eaten during pregnancy. The rapid gain in weight during the first week of pregnancy following a low level of thiamine was the result of the increase in food intake which occurred immediately when the level of dietary thiamine was increased. The weight of the young was similar with all of the diets investigated but differences in litter size were sufficient to account for differences in weight gain during the final week of pregnancy.

Chow⁸ has recently reported permanent stunting of the progeny when dietary restriction was imposed on the pregnant rat although such restriction failed to affect the reproductive system of the mother during subsequent pregnancies.⁹ According to Hafez (6), fetal weight depends upon the plane of nutrition in the last part of pregnancy rather than diet in early pregnancy. The ability of the rat to produce young of normal size in spite of a low intake of a thiamine-deficient diet prior to pregnancy is further evidence that diet during pregnancy is more important for the development of young of normal size than diet prior to pregnancy.

Although protein depletion may result in a depression in the concentration of albumin in the sera of rats (7, 8), the consistent tendency for serum albumin to parallel dietary thiamine suggests that thiamine intake contributed to the results obtained in the present study.

Differences in γ -globulin level in the sera of rats fed deficient diets prior to pregnancy also appear to be the result of vitamin intake during pregnancy. Significant differences in serum γ -globulin have been reported by other investigators when vitamin-deficient diets were fed. LaComme (9) observed a large increase in serum

⁸ Chow, B. F., and C. J. Lee. 1964. The effect of dietary restriction during pregnancy and lactation on food utilization. *Federation Proc.*, 23: 291 (Abstract).

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TABLE 3
Thiamine content of maternal liver and of fetuses

Dietary thiamine		Maternal livers			Fetuses		
Pre-pregnancy	Pregnancy	No. analyzed	Avg wt	Thiamine	No. of litters analyzed	Avg litter wt	Thiamine
mg/kg	mg/kg		g	μg/g		g	μg/g
2.5	2.5	10	12.5	4.61 ± 1.01 ¹	10	47.5	1.70 ± 0.24
2.5	5.0	7	12.6	8.42 ± 1.21	8	49.4	2.19 ± 0.13
0.5	2.5	5	11.5	4.61 ± 0.67	5	31.5	1.75 ± 0.18
0.5	5.0	11	10.4	8.46 ± 1.03	13	37.0	2.14 ± 0.12

¹ SE of mean.

γ-globulin of pigeons subjected to experimental beriberi. Increased γ-globulin in the sera of riboflavin-deficient rats was reported by Jacquot-Armand (10).

The thiamine content of maternal livers and fetuses reflects the diet fed during pregnancy rather than that fed prior to pregnancy. Liver storage and possibly fetal transfer of the vitamin were maximum with the diet containing 5 mg thiamine/kg. Morrison and Sarett (11) reported liver thiamine levels of 8.7 μg/g and 8.4 μg/g in female rats postpartum when the animals were fed a diet containing 75 mg thiamine/kg during pregnancy. These levels of liver thiamine are comparable with that of 8.4 μg/g observed in animals receiving 5 mg/kg in the present study. Everson et al. (12) reported the thiamine content of the fetus at term to be 2.4 μg/g when mothers were maintained with a diet containing 140 to 145 μg thiamine/day. In the present study, the values for fetuses averaged 2.2 μg/g. Average thiamine intakes of animals fed 5 mg/kg ranged from 76 to 93 μg/day from the first to the third week of gestation. The somewhat higher fetal thiamine level of 3.0 μg/g reported by Barrett and Everson (13) was not the result of a higher maternal thiamine intake.

In the present study apparently both levels of thiamine fed during pregnancy provide intakes which support normal reproduction in the rat receiving an adequate ration prior to pregnancy. The basal diet provided a maximum of 50 μg/day during the final week. It thus appears that, for the last 3 days of gestation, the requirements for normal reproduction in the

rat may be less than 80 μg/day as recommended by the NRC (14). The data further suggest that the dietary level of thiamine promoting maximal maternal liver and fetal thiamine reserves in the rat is probably no greater than 80 μg/day.

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EFFECTS OF VITAMIN IMBALANCE UNDER CONDITIONS OF
AD LIBITUM FEEDING AND REDUCED CALORIC INTAKE¹

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In recent years a number of papers have appeared concerning the effects of vitamin imbalance in experimental animals and man. Scandinavian workers (1-3) found that human subjects suffering from multiple dietary deficiencies developed symptoms of niacin deficiency when dosed with thiamine alone. Sydenstricker (4) demonstrated that when niacin alone was given to pellagrins certain signs of their disease, presumably due to a deficiency of other factors, were intensified. Morgan (5) observed that administration of niacin or pantothenic acid alone to dogs receiving "ample amounts of all necessary vitamins except those of the filtrate factors" resulted in a gradual loss of neuromuscular control and sometimes sudden death. All these experiments have one point in common: namely, that the administration of a vitamin resulted in the intensification of symptoms or the precipitation of pathologies that did not occur in animals not so treated. Other investigators, however, were unable to demonstrate adverse reactions following unbalanced dosing even with massive doses of the B-vitamins (6, 7). In view of Richards' suggestion that adverse effects of vitamin imbalance might only be demonstrated under conditions of stress as distinct from growth or maintenance (8), the present study was undertaken to determine the effects of unbalanced dosing both in animals fed (1) *ad libitum* and (2) under the stress of reduced caloric intake.

PROCEDURE AND RESULTS. Ninety-six female rats of the University of Southern California strain were raised to maturity on a stock ration and selected for the experiment at approximately 90 days of age and an average body weight of 170 grams (range 149 to 196 grams). Two basal rations were employed: diets A and B. Diet A was a purified ration containing the B-complex factors in synthetic form; diet B was similar in composition but contained yeast in place of the synthetic B-factors. The synthetic vitamins were incorporated in diet A in amounts corresponding to their content in yeast so that the thiamine, riboflavin, pyridoxine, niacin and pantothenic acid content of the two diets was virtually identical. Both diets A and B were supplemented with massive doses of thiamine hydrochloride, riboflavin and nicotinic acid, the three vitamins employed in the flour enrichment program; and the effects of feeding each of these diets with and without supplements were determined (table 1). Diets were prepared weekly and kept under refrigeration. Animals were fed the above

¹ The research which this paper reports was undertaken in co-operation with the Committee on Food Research of the Quartermaster Food and Container Institute for the Armed Forces. The views or conclusions contained in this report are those of the authors. They are not to be construed as necessarily reflecting the views or indorsement of the War Department.

rations (1) *ad libitum* and (2) in amounts of 6 grams per day representing a caloric restriction of approximately 50 per cent. Diets were administered daily except Sunday with portions doubled for the Saturday feeding. Animals were weighed weekly, and feeding was continued for 20 weeks (12 animals per group).

Table 1
Composition of experimental diets*

COMPONENT	DIETS A ₁ AND A ₂	DIETS B ₁ AND B ₂
	%	%
Yeast**	0.0	12.0
Vitamin test casein†	30.0	30.0
Salt mixture††	4.5	4.5
Sucrose	55.5	43.5
Cottonseed oil	10.0	10.0

Vitamin supplements added to diets		
	mgm. %	mgm. %
Thiamine hydrochloride	7.2	0.0
Riboflavin	0.9	0.0
Pyridoxine hydrochloride	1.5	0.0
Calcium pantothenate	6.72	0.0
Nicotinic acid	6.0	0.0
2-methyl-naphthaquinone	0.5	0.5
Choline chloride	120.0	120.0

Additional supplements of thiamine hydrochloride, riboflavin and nicotinic acid were added to diets A₂ and B₂ in the following amounts per kgm. of diet: thiamine hydrochloride 3.6 grams, riboflavin 0.45 gram and nicotinic acid 3.0 grams.

* All rats were fed once weekly 3 mgm. of alpha-tocopherol and a vitamin A-D concentrate (Nopco Fish Oil Concentrate assaying 800,000 U.S.P. γ of vitamin A and 80,000 U.S.P. γ of vitamin D per gram) containing 100 U.S.P. γ of vitamin A and 10 U.S.P. γ of vitamin D.

** Brewers' type yeast #200, Anheuser-Busch, Inc., St. Louis, Mo. Each gram, according to the manufacturer, contained the following vitamin potencies: thiamine, 600 μ g; riboflavin 75 μ g, pyridoxine 100-125 μ g, pantothenic acid 420-560 μ g and nicotinic acid 350-500 μ g.

† Vitamin Test Casein, General Biochemicals, Inc., Chagrin Falls, Ohio.

†† Salt Mixture no. 1, (9).

Results are summarized in table 2. Data were computed on the basis of the top 10 animals in each group with the smallest two in each series eliminated in order to minimize variations in group averages due to infection or atypical responses on the part of individual rats. At least 10 animals in each group survived the experimental period of 20 weeks. When feeding was restricted to 6 grams per day, no significant difference in body weight or gross appearance was observed either on the synthetic or yeast-containing rations between animals fed control diets and those on rations containing massive doses of thiamine hydrochloride, riboflavin and nicotinic acid. For the first 6 weeks of feeding

animals lost weight rapidly on all diets; during the next 6 weeks they continued to lose weight but at a smaller rate. During the last 8 weeks, however, animals regained in virtually all cases part of the weight they had initially lost. After 20 weeks of feeding no significant difference in body weight was observed on any of the four diets employed. On *ad libitum* feeding body weight was greater on control diets than on similar rations containing the thiamine, riboflavin and nicotinic acid supplements. Statistically these differences were not marked but a similar trend was observed on both synthetic and yeast-containing rations. Similarly gain in body weight was greater on diets B_1 and B_2 than on comparable synthetic rations.

TABLE 2

Effects of massive doses of thiamine hydrochloride, riboflavin and nicotinic acid on the body weight of rats fed ad libitum and under conditions of reduced caloric intake

DIETARY GROUP	NO. OF ANIMALS	INITIAL BODY WT. grams	AVERAGE CHANGE IN BODY WT. IN GRAMS AFTER		
			6 weeks	12 weeks	20 weeks
6 gram per day series					
A ₁	10	172.6	-38.0	-48.0	-39.3±1.1*
A ₂	10	168.0	-33.0	-44.6	-40.7±3.0
B ₁	10	170.5	-39.2	-50.2	-43.2±3.4
B ₂	10	168.0	-45.5	-59.0	-51.0±3.7
Ad libitum series					
A ₁	10	168.4	+26.9	+28.1	+42.1±7.2
A ₂	10	169.6	+16.2	+21.9	+23.4±4.1
B ₁	10	170.5	+37.0	+49.0	+60.6±5.4
B ₂	10	171.1	+19.8	+28.9	+41.9±5.2

* Standard error of the mean; $\frac{\sigma}{\sqrt{n}}$.

Effects of massive doses of thiamine hydrochloride, riboflavin and nicotinic acid on the B. M. R. of rats fed ad libitum and under conditions of reduced caloric intake. Inasmuch as thiamine, riboflavin and nicotinic acid are constituents of enzyme systems concerned in cellular oxidation and reduction, the question arose as to what effect massive doses of these vitamins might have on the basal metabolic rate of animals fed *ad libitum* or under conditions of reduced caloric intake. Accordingly, the basal metabolism was determined after 12 weeks of feeding in rats described in the previous section. The apparatus used was a closed circuit type with a capacity of 2 liters (10). Carbon dioxide was absorbed with sodium hydroxide, and oxygen consumption was determined from pressure changes recorded by means of a water manometer. The respiration chambers were kept at 28°C; readings obtained were corrected to standard temperature and pressure. Food was removed from the animals' cages the evening prior

to the metabolism test. At least six successive 5-minute intervals were recorded for each animal, with care being taken to record oxygen consumption when animal activity was at a minimum.²

Findings are summarized in table 3. When feeding was restricted to 6 grams per day, no significant difference in basal metabolic rate was observed either on the synthetic or yeast-containing rations between animals fed control diets and those on rations containing massive doses of thiamine hydrochloride, riboflavin and nicotinic acid. In all groups fed a reduced caloric intake oxygen consumption per 100 grams body weight was significantly less than for animals fed similar rations *ad libitum*. On *ad libitum* feeding, however, basal metabolic rates were greater on the control diets than for rations containing the thiamine, riboflavin and nicotinic acid supplements. Statistically these differences were not marked but they occurred on both the synthetic and yeast-containing rations.

TABLE 3

Effects of massive doses of thiamine hydrochloride, riboflavin and nicotinic acid on the B.M.R. of rats fed ad libitum and under conditions of reduced caloric intake

DIETARY GROUP	NO. OF ANIMALS	O ₂ CONSUMPTION cc/hr/100 grams BODY WT.	DIETARY GROUP	NO. OF ANIMALS	O ₂ CONSUMPTION cc/hr/100 grams BODY WT.
<i>6 grams per day series</i>			<i>Ad libitum series</i>		
A ₁	8	91.5±9*	A ₁	4	131.4±16*
A ₂	8	85.2±4	A ₂	4	116.0±6
B ₁	8	89.8±11	B ₁	4	138.0±19
B ₂	8	94.4±11	B ₂	4	112.2±10

* Average deviation.

Effects of massive doses of thiamine hydrochloride, riboflavin and nicotinic acid on the blood counts of rats fed ad lib. and under conditions of reduced caloric intake. The suggestion has been made that unbalanced vitamin therapy if sufficiently prolonged may increase requirements for other nutrients to the extent that deficiencies occur. Since leucopenia, granulocytopenia and other abnormalities in the formed elements of the blood may occur in deficiency states, the question arose as to what effect massive doses of thiamine, riboflavin and nicotinic acid might have on the blood count of rats fed *ad libitum* or under conditions of reduced caloric intake. Accordingly animals fed the diets previously described were selected after 12 weeks of feeding, and total and differential white cell counts, hemoglobin determinations and total red cell counts were made on the tail blood of all rats. Differential counts were made on smears stained with Wright's stain, 100 cells on each of two slides being employed for each analysis. All blood counts were made in duplicate.

² We are indebted to Dr. R. J. Winzler of the Department of Biochemistry and Nutrition of the University of Southern California for his assistance with the B.M.R. determinations. Readings were made by G.D.M.

No significant difference in total erythrocytes or hemoglobin levels was observed either on synthetic or yeast-containing rations between animals fed control diets and those on rations containing massive doses of thiamine hydrochloride, riboflavin and nicotinic acid. Erythrocytes averaged 7.4 to 8.1 million per cc. of blood for the various groups (range 6.7-9.6 million per cc.), with hemoglobin averaging 15.3 to 15.9 mgm./100 cc. (range 14.0-17.8 mgm./100 cc.). Data on leucocyte counts are summarized in table 4. In agreement with earlier findings (11) a significant reduction in total leucocytes was observed in animals fed reduced caloric intakes. This occurred on both synthetic and yeast-containing rations. The two diets differed, however, in respect to percentage and total granulocytes per cubic centimeter of blood. When feeding was restricted

TABLE 4
Effects of massive doses of thiamine hydrochloride, riboflavin and nicotinic acid on the leucocyte count of rats fed *ad libitum* and under conditions of reduced caloric intake

DIETARY GROUP	NO. OF ANIMALS	TOTAL LEUCOCYTE COUNT		GRANULOCYTES	
		Average*	Range	Per cent*	Total*
6 gram per day series					
A ₁	10	8,100±450	6,200-10,700	13.8±0.4	1159± 30
A ₂	10	8,290±420	7,000-10,800	14.5±1.0	1202± 85
B ₁	10	7,440±370	6,300-10,800	25.9±1.0	1927± 74
B ₂	10	7,490±620	5,000-11,000	30.4±0.7	2277± 52
Ad lib series					
A ₁	10	13,500±580	11,400-17,200	15.2±1.4	2052±180
A ₂	10	13,100±660	9,000-18,800	22.3±2.4	2921±314
B ₁	10	13,470±440	11,200-15,600	15.1±0.8	2034±108
B ₂	10	14,570±810	10,800-17,400	22.7±0.6	3307± 87

* Including standard error of the mean, $\frac{\sigma}{\sqrt{n}}$.

to 6 grams per day, the percentage of granulocytes on diet B₁ was increased so that total granulocytes per cc. of blood were equal to values observed on this same ration when fed *ad libitum*. No such increase in percentage of granulocytes occurred, however, on diet A₁ when fed under similar conditions of caloric restriction with the result that total granulocytes per cc. of blood were reduced in this series by approximately 50 per cent. No significant difference was observed at this level of feeding either on synthetic or yeast-containing rations between the granulocyte and total leucocyte count of rats fed control diets and animals fed similar rations containing massive doses of thiamine hydrochloride, riboflavin and nicotinic acid. On *ad libitum* feeding, however, percentage and total granulocytes were greater on diets containing the thiamine, riboflavin and nicotinic acid supplements than they were on control rations. Statistically these dif-

ferences were not marked but they occurred on both the synthetic and yeast-containing rations, and at least for the latter appear to be significant. Total leucocytes per cubic centimeter of blood were similar for all diets employed.

Discussion. Available data indicate the nutritional requirements of an animal may be affected by such factors as physical exertion, fever, drugs, toxins, abnormal environmental conditions, pregnancy, lactation, hyperthyroidism and related "stress factors" (12); and that caloric restriction may also serve as such a factor (11). In view of Richards' suggestion that adverse effects of vitamin imbalance may only develop under conditions of stress as distinct from growth or maintenance (8), it was felt that animals fed reduced caloric intakes might demonstrate adverse effects of unbalanced dosing more readily than animals fed similar rations *ad libitum*. Reduced caloric intake was employed as the stress factor in the present experiment since low caloric diets are widely consumed today by persons unable to obtain sufficient food to meet body requirements as well as by persons on therapeutic regimes; and it was believed desirable to determine the effects of vitamin imbalance under these conditions.

When feeding was restricted to 6 grams per day, no adverse effects were observed on either of the basal rations employed attributable to the administration of massive doses of thiamine hydrochloride, riboflavin and nicotinic acid. Gross appearance, loss in body weight, basal metabolic rate, R. B. C., Hb., total W. B. C. and differential counts did not differ significantly between animals fed control diets and those fed similar rations containing the vitamin supplements. Under conditions of *ad libitum* feeding no significant difference in gross appearance, R. B. C., Hb., and total W. B. C. count was observed between animals fed the control and vitamin-supplemented diets. The latter group, however, gained less weight, had a lower basal metabolism and a higher granulocyte count per cubic centimeter of blood than animals fed the control rations. These differences although consistent for both the synthetic and yeast-containing diets were not sufficiently marked in most cases to be statistically significant. Findings indicate, at least for animals fed reduced caloric intakes, that under conditions of the present experiment vitamin imbalance, as caused by massive doses of thiamine, riboflavin and nicotinic acid, did not result in adverse effects in the female rat.

SUMMARY

Female rats were maintained for 20 weeks on purified rations and similar diets containing massive supplements of thiamine hydrochloride, riboflavin and nicotinic acid. Two basal rations were employed: one containing the B vitamins in synthetic form and the other providing them as present in yeast. Animals were fed *ad libitum* and at a caloric restriction of 50 per cent.

Under conditions of *ad libitum* feeding no significant difference in gross appearance, R. B. C., Hb., and total W. B. C. count was observed between animals fed control diets and those fed similar rations containing the thiamine, riboflavin and nicotinic acid supplements. The latter group, however, gained less weight, had a lower basal metabolism and a higher granulocyte count per cubic centimeter of blood than animals fed control rations.

At a caloric restriction of 50 per cent no significant difference in gross appearance, body weight, basal metabolic rate, R. B. C., Hb., total W. B. C. and differential counts were observed between animals fed control diets and those fed similar rations containing the vitamin supplements.

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SYNTHESIS OF VITAMINS BY INTESTINAL BACTERIA

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Recent studies on growth factors in microorganisms have contributed much to our general knowledge of nutrition and have provided a basis for devising quantitative microbiological assays for vitamins occurring in both plant and animal materials. Deficiencies of special growth factors for yeasts, molds and bacteria have received much attention recently, but the synthesis of vitamins by these non-green plants, of equally great significance, has not been extensively investigated.

The extent to which bacteria living normally in the alimentary tract of an animal may synthesize growth factors and contribute directly to the vitamin requirements of the animal constitutes a problem of some importance. The present paper represents an attempt to determine the approximate amounts of certain B vitamins produced by species of intestinal bacteria grown as pure cultures in a chemically defined medium.

Synthesis of vitamins has already been reported for a considerable number of bacteria. Snell and Strong¹ demonstrated synthesis of riboflavin by lactic acid bacteria. Silverman and Werkman² showed that certain propionic acid bacteria make thiamine or its intermediates. Some strains of dysentery bacilli³ are able to form thiamine, and also Coenzyme I or II, riboflavin and perhaps biotin. It has been reported that a strain of diphtherial organisms⁴ can make thiamine, Coenzyme I or II and riboflavin.

The evidence obtained by several investigators indicates that bacteria normally living in the rumina of herbivores, such as sheep and cattle, produce considerable quantities of vitamins. Thus it has been found that the common *Bacillus vulgatus* living in the intestines of herbivores is capable of synthesizing thiamine.⁵ Recently it has been shown⁶ that considerable amounts of riboflavin, pyridoxine and the antihemorrhagic vitamin are formed in the rumina of sheep and cows fed on diets low in these vitamins, and the source of the vitamins is assumed to be commercial microorganisms. Almquist, et al.,⁷ demonstrated that common bacteria, such as *Bacillus subtilis* and *Escherichia coli*, can synthesize vitamin K. The phenomenon of reduction which gives protection against certain vitamin deficiencies in labo-

ratory animals indicates that the normal intestinal flora of mammals may synthesize appreciable amounts of vitamins.

The intestinal bacteria employed in the present study were kindly supplied by Dr. George Valley, Department of Bacteriology, Yale University. The organisms, *Escherichia coli*, *Proteus vulgaris*, *Bacterium aerogenes*, *Alcaligenes fecalis*, *Bacillus mesentericus* and *B. vulgatus*, were grown as stock cultures on Difco nutrient agar. A special liquid medium prepared for the studies of growth factor production was made up as follows: K_2HPO_4 , 1.0 gm.; $MgSO_4 \cdot 7H_2O$, 0.1 gm.; NaCl, 5.0 gm.; $CaCl_2$, 0.005 gm.; glucose, 10.0 gm.; recrystallized asparagine, 2.6 gm.; 1-tryptophane, 0.1 gm.; 1-cystine, 0.05 gm.; redistilled water, 1 liter. Small measured amounts of the following trace elements were added: Fe, Mn, B, Zn, Cu

TABLE 1
VITAMIN CONTENT OF BACTERIAL CULTURES GROWN FOR 48 HOURS AT 36°C. AND INOCULATED MEDIUM KEPT AT -2°C.

SPECIES OF BACTERIA	BIOTIN		RIBOFLAVIN		THIAMINE		NICOTINIC ACID	
	MILLI-GAMMA PER ML. OF MEDIUM	MILLI-GAMMA PER ML. OF CULTURE	GAMMA PER ML. OF MEDIUM	GAMMA PER ML. OF CULTURE	GAMMA PER ML. OF MEDIUM	GAMMA PER ML. OF CULTURE	GAMMA PER ML. OF MEDIUM	GAMMA PER ML. OF CULTURE
<i>Escherichia coli</i>	0	1.050	0	0.048	0.023	0.075	0.004	0.028
<i>Proteus vulgaris</i>	0	2.385	0.001	0.044	0.023	0.104	Required
<i>Bacterium aerogenes</i>	0.015	2.370	0.004	0.140	0.005	0.148	0.005	0.300
<i>Alcaligenes fecalis</i>	0.023	0.446	0	0.067	0.033	0.146	0	0.066
<i>Bacillus mesentericus</i>	Required	0	0.023	0.015	0.108	0	0.339
<i>Bacillus vulgatus</i>	0.028	1.365	0	0.136	0.031	0.150	0	1.181

and Mo. The medium was adjusted to pH 6.8 and sterilized by autoclaving at 15 lb. for 15 minutes. The inoculum was prepared by transferring a small amount of the organisms growing on agar to 5 ml. of physiological salt solution in test tubes. With a sterile pipette 0.1 ml. of the saline suspension was inoculated into 20 ml. of sterile culture liquid contained in small Erlenmeyer flasks. One set of inoculated flasks was kept as a control with growth inhibited in a room at -2°C., while the other was maintained in an incubator at +36°C. The period of growth was 48 hours unless stated otherwise.

Proteus vulgaris appears unable to synthesize nicotinic acid and *B. mesentericus* is deficient in biotin. It was found necessary, therefore, to add the deficient vitamin to the basal medium for these species in order to obtain growth so that tests could be made for the other vitamins which might be synthesized.

The methods used in assaying for riboflavin, biotin and nicotinic acid involved the use of *Lactobacillus casei* ϵ , *Saccharomyces cerevisiae* F. B. and *Lactobacillus arabinosus* as indicators in microbiological tests described by Williams and others.⁸ Thiamine activity was tested by the *Phanerochaete* assay method.⁹ Growth of the bacteria to be tested was measured with a turbidimeter, and the fresh weight of the cells in each culture was calculated by reference to standard fresh weight turbidity graphs prepared for all the species studied. At the end of the growth period, all cultures were acidified with sufficient concentrated H_2SO_4 to make the liquid approximately 1 N. The acidified cultures were autoclaved at 15 lb. pressure for 30 minutes to effect hydrolysis of the cells. The samples were cooled, brought to pH 5.0 with NaOH and diluted to standard volume. The amounts of the solutions to be used in making the tests were determined by preliminary trials, and

TABLE 2
SYNTHESIZED VITAMIN RESIDUES IN CULTURES GROWN FOR 48 HOURS AT 36°C. VALUES
EXPRESSED AS GAMMA PER GRAM OF FRESH CELLS

SPECIES	BIOTIN	RIBOFLAVIN	THIAMINE	NICOTINIC ACID
<i>E. coli</i>	2.3	106	115	82
<i>P. vulgaris</i>	3.2	57	95	None ?
<i>B. aerogenes</i>	1.1	41	43	89
<i>A. fecalis</i>	0.5	78	132	77
<i>B. mesentericus</i>	None ?	14	53	204
<i>B. vulgatus</i>	0.8	82	72	709

appropriate aliquots were employed at two concentration levels for each vitamin assay so that growth of the indicator organism would fall within a suitable range of response.

Some of the results obtained with vitamin assays performed on six species of bacteria are shown in table 1. Each value in the table represents the average of four or six determinations. The whole series of assays were repeated at different times on different cultures. In actual practice the test organisms gave satisfactorily consistent responses both to varied amounts of synthetic growth factors and to additions of bacterial extracts.

As indicated in table 1, the cultures which had grown at 36°C. for 48 hours showed a higher content of the four vitamins per ml. of fluid than did the inoculated medium in which growth was inhibited by low temperature. The results are taken to mean that under the conditions of the experiment these species of bacteria synthesize B vitamins in greater amounts than are used in their metabolism, and the residues accumulate in the cultures. The biotin, nicotinic acid, riboflavin and thiamine accumulated by the growing organisms were calculated as gamma per gram of fresh bacterial cells. These data are shown in table 2. The quantity of biotin was much lower

than the other growth factors found in the cultures. It is generally known, of course, that biotin exhibits biological activity in exceedingly small amounts. What the influence of different cultural conditions might be upon production of vitamins by bacteria would be worth further study.

An important question is how much of the total growth factors produced may be liberated from the cells into their environment. The few tests which have been made on filtrates and whole cultures of *E. coli* and *B. aerogenes* indicate that from 1 to 15% of the total biotin and nicotinic acid present in 48-hour cultures may occur outside the cells, while somewhat larger portions of the riboflavin and thiamine appear to be leached from the bacteria. In cultures of *B. aerogenes* which had grown for 111 hours, about 30% of

the biotin, thiamine and riboflavin and 40% of the nicotinic acid produced by the bacteria were found in the filtrate. Age of the cultures and other factors appear to be important in determining the distribution of these water-soluble vitamins between the microorganisms and the medium in which they live.

A study was made concerning the vitamins occurring in cultures of *B. aerogenes* at different periods of time up to 111 hours after inoculation of the basal medium. The results of this study are shown in the accompanying figure 1. It appears that the bacteria synthesized comparatively large amounts of vitamins during the early stages of growth.

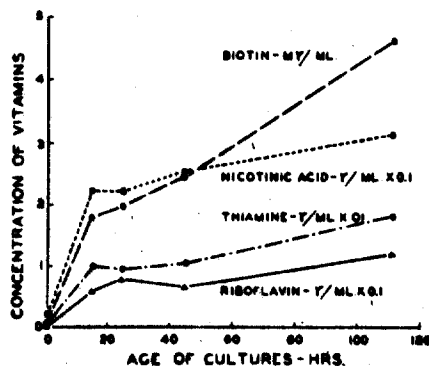


FIGURE 1

Concentration of vitamins in cultures of *B. aerogenes* grown in a chemically defined medium for different periods of time. Maximum values per ml. of culture suspension were as follows: biotin, 4.6 my; nicotinic acid, 0.31 γ ; thiamine, 0.18 γ ; riboflavin, 0.12 γ .

At 14½ hours the thiamine, nicotinic acid and riboflavin content of the cultures attained values almost as great as those reached subsequently up to 48 hours. The biotin content increased continuously throughout the entire period. From the standpoint of utilization of growth factors by the bacteria, it is significant that synthesis of the vitamins occurred early in the period of growth. Information of this kind should be valuable also in any attempt to estimate possible uses which may be made of the vitamins synthesized by bacteria.

In recent times the synthetic powers of microorganisms have been steadily assuming greater significance in relation to human welfare, as, for example, in the employment of bacteria and molds in the dairy and chemical industries and the use of yeast for the sake of its B vitamins as a supple-

ment in the diet of man. The extent to which microorganisms living normally in the alimentary tract of an animal may synthesize vitamins and contribute directly to the vitamin requirements of that animal constitutes a problem which has not yet received adequate attention. Demonstration of the synthesis of vitamins by intestinal bacteria, as presented in this report, should have considerable significance in connection with further investigations on the nutritional relationships existing among microorganisms, animals and man.

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THEORY OF THE EFFECTS OF LIGHT INTENSITY AND DURATION IN DETERMINING VISUAL RESPONSES

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In this paper are presented some applications to the phenomena of brightness discrimination and absolute threshold measurements of a theory based partly on the familiar differential equation proposed by Hecht² to account for some of the phenomena of the sensory process. A generalized form of this equation is

$$\frac{dx}{dt} = \sum_{i=1}^N \left[k_i I (a_i - x)^{m_i} - k_{-i} x^{n_i} \right],$$

where, for vision, I represents the intensity of the stimulating light, x the concentration of photoproducts broken down from the original concentration a_i of the light-sensitive substance, t represents time, m_i and n_i are integral exponents indicating the order of the reactions and k_i and k_{-i} are dimen-

COMPARATIVE TOXICOLOGY OF THIAMINE, ITS MONOPHOSPHORIC ESTER AND COCARBOXYLASE

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SUMMARY. — The acute LD_{50} of thiamine monophosphate (TMP) and thiamine diphosphate (TDP) was compared to the acute LD_{50} of thiamine (T) by means of standard bioassay techniques. Three species, mouse, rat and rabbit and two administration routes (i.v. and i.p.) were used. TMP and TDP are compared to be less toxic than T. In addition, TDP appears to be less toxic than TMP.

The comparison of the three compounds was also done by means of a prolonged administration experiment of four weeks in rats treated intraperitoneally. TMP and TDP appear to be less toxic than T, when equimolecular doses of the three compounds are used. In addition, TDP shows to be less toxic than TMP when doses of $\frac{1}{2}$ LD_{50} are used.

RIASSUNTO. — Sono state confrontate le tossicità acute della tiamina (T), del suo estere monofosforico (TMP) e della cocarbossilasi (TDP) in tre specie animali (topino, ratto e coniglio) mediante due vie di somministrazione (endovenosa ed endoperitoneale). Mediante metodi statistici appropriati si è calcolata la potenza relativa, o titolo, del TMP e del TDP, assumendo la T come standard.

I risultati ottenuti confermano la minore tossicità acuta del TMP e del TDP rispetto alla T e dimostrano la minore tossicità acuta del TDP rispetto al TMP.

Infine anche per le prove comparative di tossicità subacuta (4 settimane) nel ratto per via endoperitoneale è stato dimostrato il minore effetto tossico del TMP e del TDP rispetto alla T (trattamento con dosi equimolecolari), nonché del TDP rispetto al TMP (trattamento con dosi pari a $\frac{1}{2}$ della LD_{50}).

The acute toxicity of thiamine (T), thiamine monophosphoric ester (TMP) and thiamine diphosphoric ester (TDP) has been studied previously on various animal species (1-10).

A survey of the pertinent literature shows that the two phosphoric

esters of T have been found to be less toxic than T itself; however, most of the data are not obtained by direct comparison (4, 5); therefore the confidence limits of the potency ratio among the tested compounds cannot be established. Furthermore, there are not data on comparative toxicity for T, TMP, TDP on extended treatment.

We are reporting here, data on:

A. Comparative acute toxicity of single doses of T, TMP and TDP in three animal species (mouse, rat and rabbit) by intravenous and intraperitoneal administration;

B. Comparative subacute toxicity (4 weeks) for the three compounds in rat after i.p. administration.

Acute toxicity in rabbit has been tested by determining the threshold dose causing cardiac arrest (direct assay); in mouse and rat by the comparison of the log-dose/mortality lines (indirect assay). The terms and the statistic methods used are those reported in the F.U., U.S.P. XVII, B.P. 1963 and in the text for biological assay (11).

Materials and Methods

The following substances have been used:

- thiamine hydrochloride (*), molecular weight = 337.27 (thiamine radical content of 0.786 g/l g);
- thiamine monophosphoric monohydrochloride bishydrated ester (**), molecular weight = 446.82 (thiamine radical content of 0.636 g/l g);
- thiamine diphosphoric hydrochloride ester (**), molecular weight = 460.79 (thiamine radical content of 0.575 g/l g).

Purity controls of each product were performed.

ACUTE TOXICITY: «Indirect Assay»

Male Swiss mice, (18-21 g body weight) and male Wistar rats (95-125 g b.w.) were used. They were caged at constant temperature and humidity ($T = 24^{\circ}\text{C}$; $H = 50\%$) and fed at balanced diet (***). The products tested were dissolved in water, neutralized with NaHCO_3 , when necessary and administered intravenously or intraperitoneally at different doses, but at constant volumes: 10 ml/kg i.v. and i.p. in mouse, 4 ml/kg i.v. in rat.

The animals were controlled for 48 hrs after treatment. The calculations

(*) Pierchimica.

(**) Richardson-Merrel, S.p.A., Naples.

(***) S. Morini, Solid Diet MIL Morini.

for LD_{50} potency evaluation (ratio between equipotent doses of T and of each one of the two compounds) tested with confidence limits, were performed on IBM 360/40, with the probits method using the Quantal Assay Program (*).

ACUTE TOXICITY: «Direct Assay»

Rabbits of both sex, (1.6-2.9 kg b.w.) were used, housed at constant temperature and humidity ($T = 24^{\circ}C$; $H = 50\%$) and fed at balanced diet. The samples (standard T, TMP and TDP) were injected i.v. at constant rate (0.2 ml/minute) to three groups of five rabbits, until death due to cardiac arrest occurred. The single lethal doses are expressed in mg thiamine radical/kg of body weight. The calculations of the potency evaluation were performed after the logarithmic transformation of the single lethal doses (11).

SUBACUTE TOXICITY

The compounds were injected daily i.p. for four weeks in male rats (98-120 g initial b.w.) according to the Scheme in Table I.

TABLE I

Administ. Substance	Dose mg/kg/die as pure subst.	Dose mg/kg/die as T radical	Animals No.	Treatment Period	Dose Values
T	305	240	60	4 weeks	$\frac{1}{2} LD_{50}$ T
TMP	377	240	20	4 weeks	equimolec. with $\frac{1}{2} LD_{50}$ T
TMP	797	507	40	4 weeks	$\frac{1}{2} LD_{50}$ TMP
TDP	417	240	20	4 weeks	equimolec. with $\frac{1}{2} LD_{50}$ T
TDP	1130	650	20	4 weeks	$\frac{1}{2} LD_{50}$ TDP

Results

ACUTE TOXICITY: «Indirect Assay»

The data obtained in the acute toxicity tests expressed in mg thiamine radical are reported in Table II.

(*) We thank Dr. J. Meyer and Mrs. D. Spurlock of Biostatistics Dept. of The Wm. S. Merrell, Cincinnati (USA), for sending us their Program.

TABLE II

Acute toxicity of T, TMP and TDP expressed in mg/kg thiamine radical in mouse and in rat by i.v. and i.p. routes.
Titration of TMP and TDP as to T by the indirect method.

Animal species (adminstr. route)	Compounds	N ^a of animals	N ^a of doses	Min. dose Max dose	b	LD ₅₀ and conf. limits (P = 0.05)	b*	Parallelism (P)	Potency and conf. limits (P = 0.05)
Mouse i.v.	T	50	5	30- 99	5.81	74 (65- 86)			
	TMP	190	7	284- 567	7.33	176 (117- 512)	7.07	0.63	0.16 (0.13-0.18)
	TDP	130	7	288- 575	7.81	471 (437- 519)			0.16 (0.13-0.18)
Mouse i.p.	T	170	7	157- 394	7.65	268 (218- 293)			
	TMP	250	12	636-1598	4.78	711 (659- 760)	6.07	0.14	0.38 (0.34-0.43)
	TDP	70	7	721-1441	7.64	1083 (953-1238)			0.25 (0.21-0.29)
Rat i.v.	T	100	5	140- 222	12.52	175 (159- 194)			
	TMP	70	7	253- 714	5.51	487 (434- 548)		0.02(*)	0.36
	TDP	130	7	323- 645	6.16	488 (451- 531)			0.36
Rat i.p.	T	140	7	313- 624	8.31	481 (450- 517)			
	TMP	100	5	801-1269	6.97	1015 (911-1093)	7.82	0.27	0.47 (0.43-0.53)
	TDP	80	4	1022-1411	13.47	1301 (1192-1428)			0.37 (0.33-0.41)

b = slope of line on paper log dose-probit.

b* = slope of the common line in case the parallelism hypothesis is valid.

(*) = in this assay the parallelism hypothesis is contradicted. Therefore the potency changes according to dose; relations among LD₅₀ are given for information.

The smallest variations of the confidence limits of LD₅₀ for TMP were found in mice by the i.v. route. The largest oscillations for T were found in mice by i.v.

There is no evidence of deviations from parallelism among the lines of the three compared products in three of the four assays (mouse by i.v., mouse by i.p. and rat by i.p. route) (Table II). Therefore the potency evaluation (ratio between equitoxic doses of T and of each of the two samples) are valid.

In mice TMP and TDP administered i.v. are less toxic than T and equitoxic between themselves. The ratio between LD₅₀ of T and TMP and between LD₅₀ of T and TDP is equal to 0.16. The higher confidence limits (0.18) are far from unity, which means that the potency difference among T, TMP and TDP is highly significant.

In rat by i.v. administration the parallelism conditions among the three lines are not satisfied, therefore, an evaluation of potency cannot be given; the I.D.₅₀ for TMP and TDP show that both are less toxic than T and equitoxic between themselves. The same is valid for i.p. administration. TDP appears to be less toxic than TMP in mice, since the higher confidence limits of potency for TDP are equal to 0.29 and do not reach the confidence limits for TMP (0.34). A similar condition is found in experiments on rat, where limits are equal to 0.41 and 0.43 respectively.

ACUTE TOXICITY: «Direct Assay»

Administration i.v. to rabbits of the compounds tested and the results obtained by the determinations of the threshold-doses of lethality (Table III) confirm the lower toxicity of TMP and TDP, whose potency referred to T is 0.65 and 0.48 respectively. In both cases the higher

TABLE III

Values of the single lethal doses (cardiac arrest) of T, TMP and TDP in mg/kg thiamine radical in rabbit by i.v. route. Titration of TMP and TDP as to T.

	T (Standard)		TMP		TDP	
	Single lethal doses (mg/kg)	Log doses	Single lethal doses (mg/kg)	Log doses	Single lethal doses (mg/kg)	Log doses
	215	2.3324	591	2.7716	567	2.7536
	185	2.2672	507	2.7071	571	2.7566
	345	2.5378	776	2.8909	562	2.7497
	325	2.5119	107	2.0285	548	2.7388
	502	2.7007	287	2.4579	576	2.7579
Mean		2.4700		2.6546		2.7502
Variance		0.0299		0.0340		0.0403
•Pool• Variance		0.0240				
Potency as to T		0.65 (0.46-0.93)		0.48 (0.34-0.68)		
Potency of TDP as to TMP.				0.73 (0.52-1.04)		

confidence limit does not include the unity, thus the difference between the two samples is significant at the 5% level.

In fact TDP is less toxic than TMP (potency = 0.73), even if the higher confidence limit is slightly above the unity (1.04).

SUBACUTE TOXICITY

Administration i.p. in rats of equimolecular doses (240 mg/kg daily of thiamine radical) confirms the lower toxicity of the two esters in comparison with T itself. The mortality rate after 28 days of treatment for T is 38.3% (23/60), for TMP ($P < 0.10$) 15% (3/20) and for TDP ($P < 0.005$) 0% (0/20). TDP seems to be less toxic than TMP ($P = 0.11$) (Fig. 1 a). The i.p. administration of a dose equal to $\frac{1}{2}$ LD₅₀ gave similar results: on the 28th day the mortality with T and TMP are very similar: 38.3% (23/60) and 45% (18/40) respectively, significantly higher (in both cases $P < 0.005$) than that of TDP: 5% (1/20) (Fig. 1 b).

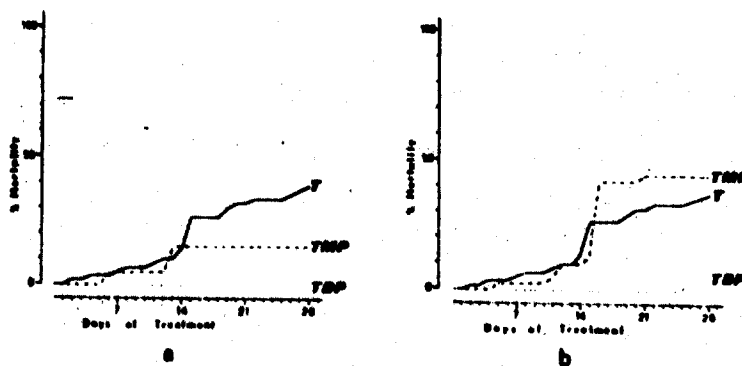


Fig. 1

a
Subacute toxicities of T, TMP and TDP administered in rat at equimolecular doses (240 mg/kg i.p. as thiamine radical). Cumulative mortality percentage as to time.

b
Subacute toxicities of T, TMP and TDP administered in rat at half doses of LD₅₀ i.p. (240, 307 and 650 mg/kg daily as thiamine radical for T, TMP and TDP respectively). Cumulative mortality percentage as to time.

Conclusions

TMP and TDP have lower acute toxicity than T, as reported by other A.A. (4, 5) and this is confirmed by our experiments. The toxicity

difference is noticed in rat, mouse (indirect assay) and in rabbit (direct assay), therefore we can conclude that the toxicity difference is not species-specific, at least as far as rodents are concerned.

Our data show a definite lower toxicity of TDP in comparison with TMP which is statistically significant in mouse and in rat treated by i.p. administration; such a demonstration in the same species by intravenous administration of the compounds, could not be conclusively obtained.

In rabbit TDP has a lower toxicity than TMP but this cannot be stated with certainty, since the higher confidence limit of the potency evaluation is slightly above the unity.

After 28 days of treatment with equimolecular doses of T, TMP, TDP, the toxicity of the three products is in decreasing order toxicity (T, TMP, TDP) in accordance with the data obtained in the acute toxicity test. Using fractions of equitoxic doses ($\frac{1}{2}$ LD₅₀) for prolonged treatment, similar results are obtained.

In fact, the mortality of the animals treated with TDP reached the 5% at the 26th day, while in the groups treated with TMP and T the mortality progressively increased to approx. 50%.

These results cannot be easily explained but they could agree with the hypothesis that, in order to penetrate the tissue structures and to exert a toxic action, TMP and TDP should be dephosphorylated first to T and that the dephosphorylation of TDP could go on at a slower rate.

Research on B₆ deficient rats suggest that the administration of TMP or TDP leads to accumulation in organs only after dephosphorylation and subsequent phosphorylation in the cells (12).

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Effect of Massive Doses of Thiamine on Fertility and Lactation in the Albino Mouse.*

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The experiments of Perla¹ showed that feeding rats with large amounts of thiamine resulted after one generation in interference with lactation, loss of maternal instinct, cannibalism, and progressive loss of fertility. Continuing this study, Perla and Sandberg² found that these symptoms were prevented by giving

manganese chloride daily to the animals. If the diet was supplemented with manganese chloride alone, interference with lactation resulted, particularly marked after one generation. The observations of Perla were confirmed by Sure.³ In contrast to the findings of Perla, and of Sure, Williams and Spies⁴ reported normal reproduction in rats kept on stock diets supplemented with large amounts of thiamine through 3 generations. The

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EFFECT OF THIAMINE EXCESS

TABLE I.
Data on Reproduction and Lactation in Albino Mice Kept on a Basal Diet of Purina Dog Chow Supplemented with (a) Thiamine, (b) Thiamine + MnCl_2 , and (c) MnCl_2 .

Generation	Supplement	No. of mice	No. of litters born	No. of litters weaned
Parent	None	10	10	8
	Thiamine	10	7	7
	" + MnCl_2	5	5	4
F_1	MnCl_2	5	5	5
	Thiamine, 1st mating	5	5	0
	" 2nd "	5	3	3
	" + MnCl_2	5	4	4
	MnCl_2	5	4	4
F_2	Thiamine	5	5	4
	" + MnCl_2	5	3	3
	MnCl_2	5	4	4

present report presents data on the effects of massive doses of thiamine in the mouse. The action of manganese was also studied.

The basal diet consisted of finely ground Purina dog chow. Albino mice of our own strain were divided into 3 groups and fed the following rations: Group 1, basal diet plus 250 mg thiamine per kilo of diet; Group 2, basal diet plus 250 mg thiamine and 1 mg MnCl_2 per kilo; Group 3, basal diet plus 1 mg MnCl_2 per kilo. The mice were placed on the diets when 21 days old, at which time they weighed 8 to 10 g. The effects of the 3 diets were studied throughout 3 generations.

The growth rate in the 3 groups was practically the same and was similar to that obtained on Purina dog chow alone. In the rat, according to Sure,³ a supplement of a large amount of thiamine has a stimulating effect on growth. The daily average intake of thiamine for the mice in Groups 1 and 2 during growth was 625-750 μg since the daily food

intake amounted to 2.5-3.0 g.

As shown in Table I, reproduction and lactation did not appear abnormal throughout the 3 generations in any of the 3 groups. Second generation mice on the thiamine-supplemented diet destroyed their young upon being mated the first time. However, a subsequent mating saw them successfully wean their young. During reproduction and especially during lactation the intake of thiamine was greatly increased due to the increased food intake. During lactation the mothers consumed as much as 8 g of diet daily. Such an amount of diet would contain 2000 μg of thiamine. The amount of MnCl_2 ingested daily during growth was approximately 3 mg, and about 8 mg during lactation.

Summary. The effect of massive doses of thiamine on reproduction and lactation in the albino mouse has been studied. No harmful action was observed.

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CONTACT DERMATITIS DUE TO THIAMINE

Report of Two Cases

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A review of the literature discloses no cases of contact sensitivity due to thiamine (vitamin B₁), and personal communication, so far as practicable, with other dermatologists fails to elicit any cases of allergic contact dermatitis due to it. Within the past two years we have observed 2 cases of such contact sensitivity. Both occurred in persons who worked in a pharmaceutical establishment, filling ampuls containing injectable materials, one of which was vitamin B₁.

The patients' sole duties in these establishments were the filling of ampuls and vials, without actually handling the contents, although from time to time the contents splashed onto their fingers and hands. The interval of time elapsing between the initial handling of the substances and the appearance of a dermatitis was three months and eight months, respectively. In each instance the original site of involvement was on the back of the hands and fingers, with subsequent spread to the wrists and forearms. Reactions to patch tests were positive in both patients and remained so for many months. Similar patch tests with the contents of other ampuls individually and collectively elicited negative reactions. Additional evidence that vitamin B₁ was responsible was the immediate recurrence of the dermatitis when the patient returned to the same work, with reexposure to the contactants.

CASE 1.—A. M., a 35 year old woman, had been employed filling ampuls and vials with various vitamin fractions since February 1948. She appeared at our office in October of that year complaining of violent itching of the fingers, hands, wrists and forearms. Her specific duty at the plant was the operation of a machine filling vials and ampuls. She never wore gloves or any other protective clothing, and so from time to time some of the ingredients spilled over her hands.

There were patches of an erythematovesicular dermatitis on the fingers, backs of the hands, wrists and forearms. Many of the patches were covered with finescales, while others showed punctate excoriations due to scratching. The patient added, on direct questioning, that at various times she filled vials with a crude liver extract under ultraviolet radiation. She felt that her hands became worse at these times and initially thought that possibly all her troubles were from this source. However, tests with crude liver (from her establishment) were done by painting the crude liver extract in full strength on the skin adjacent to the involved areas and simultaneously exposing the areas to intense ultraviolet radiation. There was neither an exacerbation of the involved areas nor an excessive reaction in the irradiated areas forty-eight hours later. Patch tests with all the ingredients used in filling the vials and the ampuls were then made. Within forty-eight hours at each of the sites of patch tests for sensitivity to vitamin B₁ there was a positive reaction especially to pure vitamin B₁, which elicited about a 3 plus reaction. These tests were repeated, along with an additional one with pure thiamine hydrochloride U.S.P. Tests were also made on controls. Forty-eight hours later the patient showed 3 plus reactions on all sites. The reactions on the controls were negative.

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Twelve days later the patch tests with vitamin B₁ were repeated, and the same 3 plus reactions were elicited. On the thirty-eighth day the same procedure was again repeated, with duplication of results.

With a view to finding some previous exposure to vitamin B₁, the patient was questioned carefully as to the use of vitamins, injections of vitamin B₁ in particular. She stated that she had not taken vitamins in any form as medicine and had not taken any other medicament for many years.

She made an uneventful recovery with routine local care, which included application of ointments and grenz radiation. At each visit the original sites of the patch tests still showed evidence of positive reactions. Some of the tested areas were covered subsequently with fine scales.

Several weeks after discharge she came back with a similar dermatitis. She had returned to the plant and resumed the filling of vials and ampuls with vitamin B₁. Simultaneously there was some erythema at the sites of the patch tests done on the occasion of her initial dermatitis. At this time she was again retested and found sensitive to all contactants containing vitamin B₁. Tests with all other ingredients elicited negative reactions.

CASE 2—C. F., a 36 year old woman, was employed at the same plant doing the same type of work as the previous patient. After only three months at this occupation she noted itching of her fingers and hands. This became progressively worse, but she continued to fill the vials with vitamin B₁. Both eyelids were swollen and red; she recalled wiping her eyes with her hands while working. On direct questioning she also stated that she had never taken vitamin B₁ by injection or ingestion or that she had ever been employed doing similar work, filling ampuls or vials, previous to her present job.

Examination disclosed patches of an erythematovesicular dermatitis on the fingers, with inflammatory edema of both eyelids. Patch tests with all the ingredients she handled were applied, but only those containing vitamin B₁ elicited positive reactions. A patch test with pure thiamine hydrochloride U.S.P. elicited a 2 plus positive reaction. The patient made an uneventful recovery and was warned about her sensitivity to substances contacted in this occupation.

Several weeks later she returned to the office with a recurrence. The dermatitis now extended to the wrists, forearms and cubital regions. She had not followed advice and had again been operating the same machine. Within the same week all her symptoms had exacerbated. Patch tests were repeated, with identical results. Following admonition not to return to the same job and to avoid contact with solutions of vitamin B₁, she has since remained well.

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declined slowly. Heating the hypothalamus for less than 3 min. or to less than 41.5° C. produced only a very small increase in evaporation, although a rise in the skin temperature of the ears occurred and respiratory rate increased.

When the hypothalamus was repeatedly heated to 41.5° C. for 3 min. at intervals, the rate of evaporation from the skin increased progressively (Fig. 1), but the magnitude of the increase tended to decline with successive stimulations.

The results of the present work demonstrate that evaporation from the skin as well as vasodilatation and panting can be stimulated by heating the hypothalamus. It is not, however, suggested that a rise in the temperature of the hypothalamus is a necessary condition for an increase in loss of moisture from the skin.

The increase in evaporation may be related to the active secretion of the sweat glands, or to an increase in diffusion of water through the skin. The progressive increase in evaporation after repeated heating is, however, difficult to account for if evaporative loss is due to simple diffusion.

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Effect of Added Vitamins on the Phagocytic Activity of the Reticulo- Endothelial System of Mice

We have reported that oestrogens stimulate the reticulo-endothelial system in the spleen, liver and lymph nodes and lead to mobilization of the phago-

cytes into the blood stream and thus probably to an increase of the general defence of the body against infection¹. We have also shown that antibiotics and sulphonamides² have little effect on the phagocytes; but cortisone depresses the reticulo-endothelial system and also reduces the concentration of gamma-globulin in the serum to about half the normal level³. Unpublished research in this laboratory also shows that when the reticulo-endothelial system is stimulated with, for example, diethylstilboestrol, the serum gamma-globulin is increased to about twice the normal level, and the formation of antibody is increased about eight times. Groups of mice and guinea pigs may be protected by this means against challenge with a number of lethal doses of virulent bacteria. Further unpublished results show that, when animals are given a diet free from vitamin A, the reticulo-endothelial system becomes greatly depressed and when normal diet (Oxo mouse and rat diet) is resumed the phagocytic index returns to normal in about thirteen days.

The present research was planned to ascertain the effect of added vitamins on the phagocytic activity of the reticulo-endothelial system in normal healthy animals.

Two hundred and ten male white mice (T.O. Swiss strain) of 20-25 gm. weight were used in these experiments. Ten animals were used to test each vitamin. The vitamins used, the dose, route of administration, frequency and duration of dosage are shown in Table 1. The estimated daily vitamin requirements, also shown in Table 1, are those given by Spector⁴.

The doses in most of the experiments are greater than the estimated requirement and during the treatment the animals remained on normal diet. Vitamin D was dissolved in olive oil, vitamin E and vitamin K (natural) in arachis oil to which a small amount of 'Teepol' was added, and the remaining vitamins were given in aqueous solution suitably buffered for stability according to information kindly supplied by Roche Products. All the solutions were made up so that one dose was contained in 0.2 ml. The route

Table 1. EFFECT OF ADDED VITAMINS ON THE PHAGOCYTIC ACTIVITY OF THE RETICULO-ENDOTHELIAL SYSTEM

Vitamin used	Estimated daily requirement	Dose	Route	Frequency of dosage	Duration of dosage	Mean phagocytic index (A value) ± S.E.
Vitamin A palmitate	20 I.U.	70 I.U. 70 I.U. 70 I.U.	I.P. I.P. I.P.	2/week 2/week 2/week	1 week 2 weeks 3 weeks	14 ± 2.6 14 ± 1.6 12 ± 1.6
Calciferol	2.0 I.U.	10 I.U. 10 I.U.	oral oral	2/week 2/week	1 week 3 weeks	11 ± 2.0 11 ± 1.0
Vitamin A + calciferol		Vit. A—20 I.U. Calciferol—2 I.U. ..	I.P. oral ..	daily daily	2 weeks 4 weeks	17 ± 2.0 16 ± 8.0
Ascorbin	0.02 mgm.	0.06 mgm.	oral	daily	1 week	17 ± 2.1
Pyridoxine	0.02 mgm.	0.08 mgm.	oral	daily	1 week	19 ± 3.1
Nicotinic acid	0.1 mgm.	0.2 mgm.	oral	daily	1 week	14 ± 1.3
Inositol	?	40.0 mgm.	oral	daily	1 week	18 ± 1.5
Biotin	0.0008 mgm.	0.006 mgm.	oral	daily	1 week	11 ± 0.6
Pantothenol	0.2 mgm.	1.0 mgm.	oral	daily	1 week	15 ± 0.5
Choline	4.0 mgm.	40.0 mgm.	oral	daily	1 week	20 ± 1.8
Cyanocobalamine	0.0008 mgm.	0.0016 mgm.	I.P.	daily	1 week	17 ± 1.2
Ascorbic acid	?	1.0 mgm.	oral	daily	1 week	15 ± 1.2
α-Tocopherol	0.1 mgm.	2.0 mgm.	oral	daily	1 week	16 ± 1.2
Methylaphthylthylne di(dihydrogen) phosphate	0.02 mgm.	0.4 mgm.	oral	daily	1 week	15 ± 1.6
Naphthaquinone	0.02 mgm.	0.4 mgm.	oral	daily	1 week	15 ± 1.0
Aneurine		0.4 mgm.	oral	daily	1 week	17 ± 1.7
Riboflavin		0.8 mgm.				
Pyridoxine		0.4 mgm.				
Nicotinic acid		2.0 mgm.				
Ascorbic acid		1.0 mgm.				
Controls						15 ± 2.0

of administration was either intraperitoneal or by stomach tube.

Two days after completion of the vitamin treatment the phagocytic activity was measured in each group of animals by the rate of disappearance of a known amount of specially prepared carbon from the circulating blood¹, the procedure used being that described in a previous communication², and the total body phagocytic activity or phagocytic index being denoted by the symbol *K*.

Ten control animals, receiving no treatment, showed a phagocytic index or *K* value of 15 ± 2.0 .

The results are shown in Table 1. In the doses administered the vitamins given to normal healthy growing mice had no discernible effect on the phagocytic activity of the reticulo-endothelial system. The above results might well have been expected since, for the most part, vitamin action appears to be related to the enzyme systems of normal cellular metabolism. In contrast, in infection, when the metabolism of the reticulo-endothelial cells increases, the requirement for the various vitamins may also be raised.

In these investigations we gratefully acknowledge advice and gifts of vitamins from Roche Products, Ltd., and Glaxo Laboratories, Ltd., and also financial assistance from the Tobacco Manufacturers' Standing Committee and the Medical Research Council.

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Effect of Bradykinin on Uterine Activity

It was reported recently¹ that plasma kinin is formed spontaneously in human plasma collected during labour. Tested on the isolated rat uterus, the maximum oxytocic activity was stated to be equivalent to several units of oxytocin per millilitre of plasma. It was suggested that kinin formation may be related to the powerful contractions of the human uterus during parturition.

As yet the only kinin which has been chemically defined and is available in a pure form is bradykinin. We therefore investigated the uterine effect of synthetic bradykinin² on the human uterus *in situ* (external^{3,4} and internal⁵ tocography) and *in vitro*⁶, and on the uterus *in situ* of anesthetized non-pregnant rats, cats⁷ and rabbits⁸.

Single intravenous injections of up to 2 $\mu\text{gm.}$ *kgm.* bradykinin and intravenous infusions of 0.14, 0.28 or 0.56 $\mu\text{gm./kgm. min.}$ of bradykinin—each dose infused for a period of 5 min.—had no oxytocic effect on the human uterus *in situ* a few hours before or after labour. In one case, 1.4 $\mu\text{gm./kgm. min.}$ infused for 3 min. was followed by a contraction, which may have been connected with the administration of the peptide. Although no uterine effect could be detected, the administration of higher doses of bradykinin

elicited a number of symptoms, such as lowering of the systemic blood pressure, hot flushes, sensation of warmth in various parts of the body, etc.

Isolated strips of human myometrium obtained at Caesarian section contracted only after bradykinin doses as high as several hundred or even thousand $\mu\text{gm./l.}$ Some strips failed to respond even to 10,000 $\mu\text{gm./l.}$, although their sensitivity to oxytocin ('Syntocinon^R'), for example, 25–100 milli-units/l. was normal (100 milli-units oxytocin is equal to 0.2 $\mu\text{gm. peptide.}$)

The rat uterus *in situ* usually gave a short-lived contraction with 100 $\mu\text{gm./kgm.}$ bradykinin (intravenous); 10 $\mu\text{gm./kgm.}$ elicited a response only in a few animals. 100 milli-units/kgm. oxytocin consistently produced a response in all the rats.

In the cat uterus *in situ* 100 $\mu\text{gm./kgm.}$ bradykinin (intravenous) invariably elicited a short-lived response. In many animals 33 $\mu\text{gm./kgm.}$ or 10 $\mu\text{gm./kgm.}$ was also effective, and one responded to as little as 3.3 $\mu\text{gm./kgm.}$ All the cats displayed a characteristic uterine reaction to 40–80 milli-units/kgm. of oxytocin.

The sensitivity of the rabbit uterus *in situ* to bradykinin varied greatly. Whereas in some rabbits the intravenous injection of 1 or 10 $\mu\text{gm./kgm.}$ was followed occasionally by a brief contraction of the uterus, others did not react even to 100 $\mu\text{gm./kgm.}$ (intravenous). All rabbits showed a normal sensitivity to methyl-ergometrine ('Methergin^R'): 0.15 mgm./kgm. invariably elicited the usual uterotonic reaction.

The isolated rat uterus is known to have a very high sensitivity to bradykinin: a minute dose of 0.00003 $\mu\text{gm./ml.}$ will sometimes elicit a contraction⁹. However, the findings presented in this communication show that bradykinin can scarcely be regarded as oxytocic in the usual sense of the word. The doses of bradykinin required to elicit a contraction of the rat, cat and rabbit uterus *in situ* are usually much higher than those evoking a marked fall of blood pressure. Moreover, the contraction is of a shape different from that produced by a low dose of oxytocin. The amounts of bradykinin which contract the isolated human myometrium are extremely high, and on the human uterus *in situ* bradykinin is virtually devoid of any effect, even in doses producing considerable distress.

These findings do not favour the view that bradykinin might be a physiological oxytocic factor responsible for increased uterine activity during labour in the human being.

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The Relation between Thiamine, Biotin and Tryptophan Metabolism, Studied in the Rat

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Previous papers (Dalgliesh, 1952; Charconnet-Harding, Dalgliesh & Neuberger, 1953) have shown that useful information on the relationship of B-vitamins to tryptophan metabolism may be obtained by examining urinary metabolites excreted after ingestion of tryptophan by vitamin-deficient animals. The present paper describes a continuation of this work with an elaboration of the technique. The effects of deficiencies of thi-

amine and biotin have been investigated. Thiamine deficiency has been known for some time to decrease the conversion of tryptophan into nicotinic acid derivatives by the rat (Junqueira & Schweigert, 1948), but there has been no evidence to show at what stage in the conversion thiamine functions. A preliminary survey of other B-vitamins suggested that pantothenic acid, nicotinic acid and biotin had no effect on tryptophan metabolism.

in the rat. This agreed with the known lack of effect of pantothenic acid on nicotinic acid formation from tryptophan by the rat (Junqueira & Schweigert, 1948). Moreover, should nicotinic acid, which is derived from tryptophan, be closely linked with tryptophan metabolism, it might be expected that a nicotinic acid deficiency once established should undergo an auto-intensification, which has never been observed. However, Shanmuga Sundaram, Tirunarayanan & Sarma (1954) have recently claimed that biotin is concerned in an early stage of tryptophan metabolism in *Neurospora*. The effect of biotin deficiency on tryptophan metabolism in the rat was therefore reinvestigated under more rigorous conditions. The work described in this paper has been the subject of two preliminary communications (Dalglish, 1954, 1955b).

METHODS

Animals. White rats, recently weaned, were obtained as required from the stock of the Postgraduate Medical School. These were kept throughout in wire-bottomed metabolism cages to prevent coprophagy. Diet was fed *ad lib.* except when tryptophan was administered. For this purpose, DL-tryptophan (usually approx. 1 mg./g. body wt./day for 1 or 2 days) was mixed in about half the usual amount of diet, in order to encourage complete consumption. The drop in weight, frequently observable in the growth curves after feeding tryptophan, is probably to be attributed to the temporary restriction of food rather than to any effect of the tryptophan *per se* though the palatability may be somewhat reduced. Water was freely available at all times. Acetic acid was added as preservative to the urine receivers.

Examination of metabolites. This was carried out by methods reported in detail elsewhere (e.g. Dalglish & Tekman, 1953; Dalglish, 1955a). Briefly, the urine was centrifuged, metabolites in the supernatant were adsorbed on deactivated charcoal, eluted with aqueous phenol, concentrated in a vacuum with simultaneous removal of phenol; the residues were submitted to paper chromatography, using butanol-acetic acid as solvent, and suitable colouring reagents applied (Dalglish, 1952, 1955a).

Thiamine-deficient regimes. **Diet 1.** Casein (ethanol-extracted for 3 days), 10%; sucrose, 36%; starch, 40%; fat mixture (4 parts arachis oil to 1 part cod liver oil, w/w), 10%; salt mixture (cf. Dalglish, 1952), 4%. The following vitamins were added to each kg. diet for the control animals [in the deficient groups the appropriate vitamin(s) were omitted]: thiamine, 10 mg.; riboflavin, 10 mg.; pyridoxine, 10 mg.; nicotinic acid, 10 mg.; p-aminobenzoic acid, 25 mg.; Ca pantothenate, 50 mg.; inositol, 100 mg.; folic acid (pteroylglutamic acid), 2 mg.; biotin, 0.1 mg.; vitamin K (Synkavit, Roche Products Ltd.), 1 mg. Each day 50 mg. rat of choline chloride were mixed in the diet as a 5% (w/v) solution. Each rat was individually given daily by dropper a solution of vitamin B₁₂ (Cytamen, Glaxo Laboratories Ltd.) equivalent to 0.05 µg./rat/day, and also 0.1 ml./day of a 1% (w/v) solution of α-tocopherol in arachis oil.

Diet 2. This was as for Diet 1, except that the amounts/kg. diet of certain vitamins were altered to the following: riboflavin, 40 mg.; nicotinic acid, 100 mg. Choline was decreased to 5 mg./rat/day.

Diet 3. This was as for Diet 1, except that B-vitamins were given in solution by stomach tube instead of being mixed in the diet. Stock solutions were made up as follows: thiamine, 45 mg./15 ml. 0.01N-HCl; pyridoxine hydrochloride, 36 mg./15 ml. 0.01N-HCl; biotin, 1 mg./10 ml. 50% (v/v) ethanol; Ca pantothenate, 326 mg./100 ml. water; p-aminobenzoic acid, 1 g./200 ml. 5% (v/v) ethanol; a mixed solution of 1 g. inositol, 1 g. nicotinic acid and 3 g. choline chloride in 200 ml. 0.1N-HCl; folic acid, 5 mg./100 ml. 50% (v/v) ethanol. The vitamin mixture for administration by stomach tube was made by adding 20 mg. riboflavin to a mixture of stock solutions as follows: p-aminobenzoic acid, 50 ml.; inositol-nicotinic acid-choline, 50 ml.; thiamine, 1.6 ml.; pyridoxine, 2.8 ml.; pantothenic acid, 16.6 ml.; biotin, 1 ml.; folic acid, 20 ml. The mixture was then made up to 500 ml. with water and stored in the cold and dark; 1 ml./day was given to each rat. Vitamin K (Synkavit) and α-tocopherol were given in arachis oil from a dropper (0.1 ml./rat/day containing 1 mg. α-tocopherol and 1 µg. Synkavit). Appropriate vitamin(s) were omitted from the deficient groups.

Biotin-deficient regime. The ethanol-extracted casein of Diet 3 was replaced by an equal weight of dried egg albumin (G. T. Gurr Ltd., London, S.W. 6). To the solid diet, before mixing to a thick paste with water, was added 1% (w/w) succinylsulphathiazole. The vitamin mixtures were the same as in Diet 3, except that the quantity of folic acid was doubled, and appropriate vitamin(s) were omitted from the deficient groups.

RESULTS

During this work various basal vitamin mixtures have been tried, and two modes of vitamin administration compared. In previous work (Dalglish, 1952; Charconnet-Harding *et al.* 1953) vitamins have been mixed in the diet. It was suggested to the author by Dr A. M. Copping that more satisfactory results were obtainable if vitamins were given daily in solution by stomach tube. The amount of vitamin consumed is then known with more certainty and, as no allowance need be made for incomplete consumption of food, there is useful economy. The comparisons made in this work have convinced the author that there are considerable advantages in Dr Copping's technique.

Five experiments were carried out in the thiamine-deficiency investigations. All gave the same results, described below, from the point of view of tryptophan metabolism, but growth curves for only two of these experiments are reproduced. Fig. 1 shows curves obtained in an experiment with Diet 1, and illustrates a hazard sometimes encountered when vitamins are mixed in the diet. It will be seen that, after 25 days, growth of the control animals markedly deteriorated. Satisfactory growth of control animals had previously

been obtained on a similar diet, and the work of Karolus & Beumann (1953) and Rombouts (1953) on the decomposition of thiamine in laboratory diets makes it probable that the poor growth of the control rats shown in Fig. 1 was due to a thiamine deficiency. In case a vitamin imbalance was involved, another group of animals were given Diet 2, in which the proportions of the

customed to the procedure. Control animals on regimes having the vitamins mixed in the diet showed a higher excretion of tryptophan metabolites after supplementary tryptophan than did animals on a stock diet or those receiving their vitamins by stomach tube, suggesting that in the former case the vitamin intake was less balanced

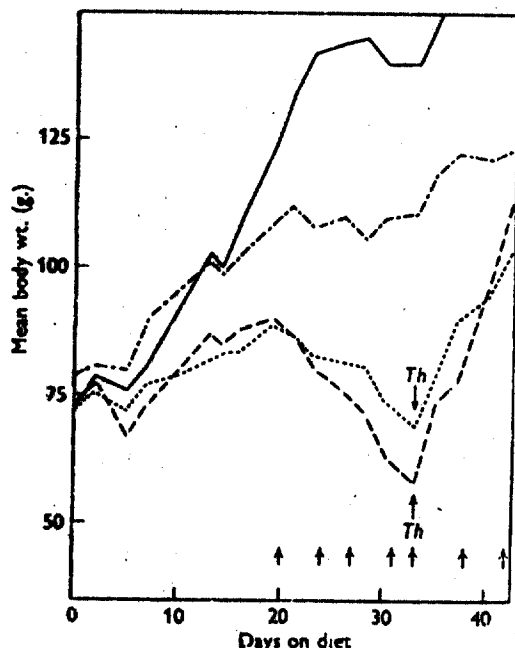


Fig. 1. Growth curves of control (—), thiamine-deficient (---), pyridoxine-deficient (-.-.-) and thiamine-pyridoxine-deficient (.....) female rats fed by the vitamins-in-the-diet technique. Each curve is the mean for three animals. Thiamine was given to the deficient animals from the points marked Th. Small arrows at the bottom represent supplementary tryptophan in the diet.

vitamins were altered. Growth of the control animals was now comparable to that found for black-and-white rats by Copping, Crowe & Pond (1951), who gave their vitamins by stomach tube, but the net consumption of vitamins on Diet 2 was much higher than in the experiments of Copping *et al.* It seemed likely that thiamine decomposition and possible similar complications would be less likely to arise when using Dr Copping's technique, described under Diet 3. Growth curves obtained on such a regime are shown in Fig. 2. Growth of the control animals was more satisfactory than when the vitamins were mixed in the diet; moreover, once practice had been obtained, administration of vitamins by stomach tube was quicker and less troublesome, the animals easily becoming ac-

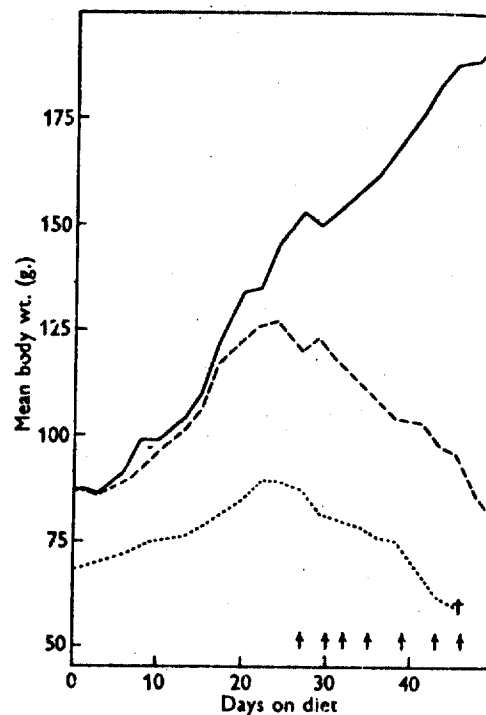


Fig. 2. Growth curves of control (—), thiamine-deficient (---) and thiamine-pyridoxine-deficient (.....) female rats given vitamins by the stomach-tube technique. Each curve is the mean for five animals. Small arrows at the bottom represent supplementary tryptophan in the diet. Animals had to be killed at the point marked †.

The results of feeding tryptophan to the various groups of animals were the same in all experiments. At the tryptophan dose level used, the control animals normally excreted small but detectable quantities of tryptophan metabolites. Tryptophan administration was generally started about 3 weeks after initiation of the dietary regime. At this stage excretion by the thiamine-deficient and control rats was indistinguishable, and the rats deficient in thiamine and pyridoxine showed a typical pyridoxine-deficient excretory pattern. As thiamine deficiency became established, as shown by the growth curves, the small excretion of tryptophan metabolites by the tryptophan-fed thiamine-deficient rats ceased. Simultaneously, in the rats

deficient in thiamine and pyridoxine, excretion of the metabolites typical of pyridoxine deficiency decreased and, by the time the animals approached their starting weights, metabolite excretion was confined to a relatively small output of xanthurenic acid. However, kynurenine fed at this stage resulted in a large output of metabolites typical of pyridoxine deficiency. There was no evidence of formylkynurenine excretion on giving tryptophan to animals with a simple thiamine deficiency. On giving thiamine to deficient animals there was a dramatic growth response, as shown in Fig. 1. Simultaneously, the typical pyridoxine-deficient excretory pattern returned in those animals previously doubly deficient.

Typical excretory patterns after establishing both thiamine and pyridoxine deficiencies are summarized in Table 1. It should be emphasized that the metabolites under discussion in this paper are those related to transformation of tryptophan into nicotinic acid. All animals, deficient or otherwise, described in this paper excreted after tryptophan administration appreciable amounts of indolic compounds. The major ones were tryptophan, indican, and two spots moving fast in butanol-acetic acid, the slower moving of which is considered to be principally indole- β -aceturic acid and the faster moving indoleacetic acid, together with indole derived from faecal contamination of the urine. In the biotin-deficiency experiments, in which succinylsulphathiazole was fed, the indole-aceturic acid spot was smaller than in the other experiments. The indole-indoleacetic acid spot was less markedly decreased.

Several diets were tried in the biotin-deficiency experiments. Biotin-deficient casein-based diets with added aureomycin (vitamins mixed in the diet) gave a slight deficiency as judged by growth curves, but the degree of deficiency was considered quite inadequate for the investigation. Diets with egg albumin (containing the biotin antagonist avidin) as protein source were tried by both the vitamins-in-the-diet and vitamins-by-stomach tube techniques. Somewhat greater deficiencies were obtained, but again these were considered inadequate. By combining supplementary succinylsulphathiazole with a diet based on egg albumin, marked deficiencies were obtained readily. These results contrast with those of Emerson & Wurtz (1944), who found an egg-albumin diet to be adequate to produce a high degree of biotin deficiency, which was not increased by succinylsulphathiazole. The amounts of succinylsulphathiazole metabolites excreted were not sufficient to interfere with chromatography of the tryptophan metabolites.

Growth curves obtained with the combined egg albumin-succinylsulphathiazole regime are shown

in Fig. 3. The biotin-deficient group showed appreciable deficiency (thinning of hair, scale formation) after 4 weeks. Scaly dermatitis, 'spectacle eyes' and 'kangaroo gait' were apparent by the sixth week and marked after the eighth week. The biotin-deficient rats had lost

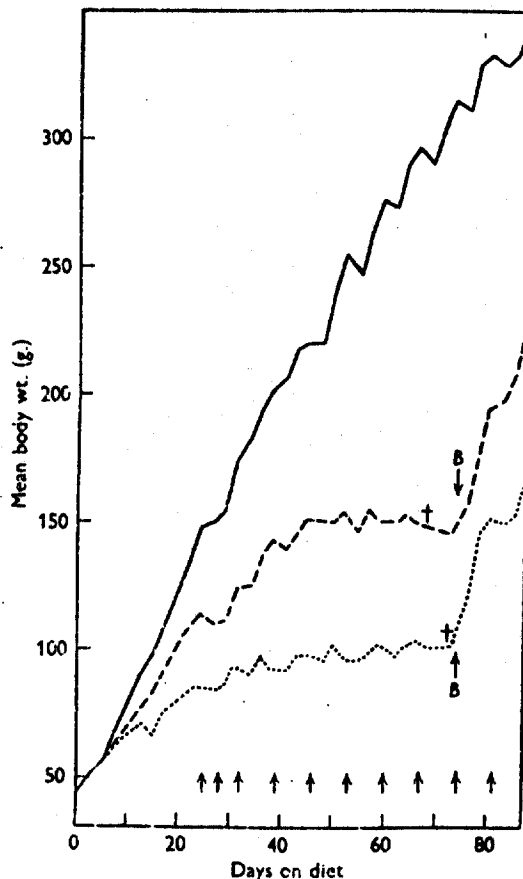


Fig. 3. Growth curves of control (—), biotin-deficient (---), and biotin-pyridoxine-deficient (.....) male rats fed on vitamins by the stomach-tube technique. Each curve is the mean for three animals. Small arrows at the bottom represent supplementary tryptophan in the diet. An animal died at the points marked †. Biotin was given to the deficient animals from the points marked B.

almost all their hair by the tenth week and at about this stage some oedema became evident. In the group deficient in biotin and pyridoxine the loss of hair was less marked, but the dermatitis was more severe and the eyes became completely closed. The control group, differing from the biotin-deficient group only in receiving biotin, grew well throughout, and the picture was therefore not complicated by any accompanying folic acid deficiency.

Table 1. *Typical metabolite-excretory patterns after feeding tryptophan to control and vitamin-deficient rats.*Excretion represented by +; metabolites sometimes excreted by \pm .

Metabolite	Non-deficient	Thiamine-deficient	Pyridoxine-deficient	Thiamine-pyridoxine-deficient	Biotin-deficient	Biotin-pyridoxine-deficient
Kynurenine	\pm	-	++	\pm	\pm	++
Kynurenine conjugates	-	-	++	\pm	-	++
Hydroxykynurenine	-	-	++	\pm	-	++
Hydroxykynurenine conjugates	-	-	+++	\pm	-	+++
Xanthurenic acid (4:8-dihydroxy-quinoline-2-carboxylic acid)	+	-	++++	+	+	++++
Anthranilic acid	\pm	-	-	-	\pm	-
Anthranilic acid conjugates	\pm	-	-	-	\pm	-

Administration of biotin to the deficient animals caused a dramatic response in growth (Fig. 3) and a noticeable growth of hair in the denuded animals within 24 hr.

Throughout the experiment no appreciable differences could be seen in the tryptophan-metabolite excretion of biotin-deficient and control rats, and at no stage did there appear to be any change in the typical pyridoxine-deficient excretory pattern of the group deficient in biotin and pyridoxine. These results are also summarized in Table 1.

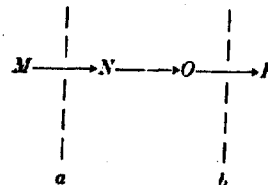
DISCUSSION

Investigations on intermediary metabolism in the intact animal can have the advantage over enzymic techniques that reactions may be revealed which are brought about by enzymes too unstable to lend themselves readily to *in vitro* investigations, and that reaction chains may be deduced from the occurrence of some intermediate which could not readily be deduced in an isolated or reconstituted system. Moreover, even if the amount and activity of an enzyme in a given organ are known, its importance in the overall picture may be difficult to assess, other than by a study of end products, as the available enzyme cannot be fully efficient unless provided with the right precursor in the right place at the right time. *In vitro* and *in vivo* experiments are thus complementary, and the remarkable recent advances in enzymic techniques do not reduce the need for experiments on the intact animal.

The approach used in these experiments is analogous for mammals to the study of deficient mutants in micro-organisms. It depends on the function of many B-vitamins as constituents of coenzymes. In a deficiency of such a vitamin an enzymic reaction for which it provides a coenzyme may be inhibited. If one reaction in a metabolic chain is inhibited and a substance prior to it in the chain is administered to the animal, it is to be expected that the substrate of the inhibited reaction (and/or simple derivatives) may be

excreted. Such an approach has led to useful information on the function of pyridoxine (Dalgliesh, 1952) and riboflavin (Charconnet-Harding *et al.* 1953) in tryptophan metabolism. The method, of course, has limitations. Thus if a vitamin deficiency results in a definite change in a pattern of metabolite excretion, definite conclusions may be drawn. But if there is deficiency, as shown perhaps by the growth curve, but no change in the pattern of metabolite excretion, it cannot be concluded that that vitamin is not concerned in that metabolic chain. The coenzyme in which the vitamin participates may be concerned in many reactions involving enzymes which have widely differing affinities for the coenzyme. In a deficiency, these reactions are not necessarily inhibited to the same degree. For example, pyridoxal phosphate is the coenzyme in transamination reactions among many others, but transamination can be apparently unaffected by a degree of vitamin B₆-deficiency which would result in marked inhibition of other vitamin B₆-dependent enzymes (cf. Snell, 1953).

An improved technique is described in this paper which is useful in certain cases. If we have a metabolic chain and a block occurs at stage *b*, then



excretion of *O* after administration of *M* has obvious significance. But it is not necessarily justifiable to deduce from an absence of metabolite excretion that there is a block at *a*. However, if blocks at *a* and *b* are combined, formation of *O* should be inhibited by block *a*, so that the excretion of *O* previously observed with a simple block at *b* should no longer occur. Thus the reality of a block at *a* can be checked.

The general outline of the pathway for conversion of tryptophan into nicotinic acid is summarized in Fig. 4 (for collected evidence see review by Dalglish, 1955c). Pyridoxine is concerned at the stage marked *C ... C* (Dalglish, 1955) and riboflavin probably at the stage marked *B ... B* (Charoonast-Harding *et al.* 1953). The present results show that thiamine deficiency inhibits formation of the substrate of the pyridoxine-dependent reaction. It must therefore function in the conversion of tryptophan into formylkynurenine, stage *A ... A* in the diagram, or in the formation of kynurenine from formylkynurenine. The latter is inherently less probable and is excluded by the absence of formylkynurenine excretion by the thiamine-deficient animals.

study of other pathways by forcing metabolism to occur by alternative routes. There is of course no evidence that thiamine deficiency does not also affect these other pathways, and a complication may also arise if an increased blood α -keto acid level causes an abnormal degree of indolepyruvic acid formation by transamination.

The results obtained in biotin deficiency strongly suggest that in the rat biotin does not influence any stage in the conversion of tryptophan into hydroxykynurenine. The results thus disagree with the conclusions of Shanmuga Sundaram *et al.* (1954) on the function of biotin in tryptophan metabolism in *Neurospora*, in which they consider biotin to be a cofactor in formylkynurenine formation. There remains, however, the possibility, con-

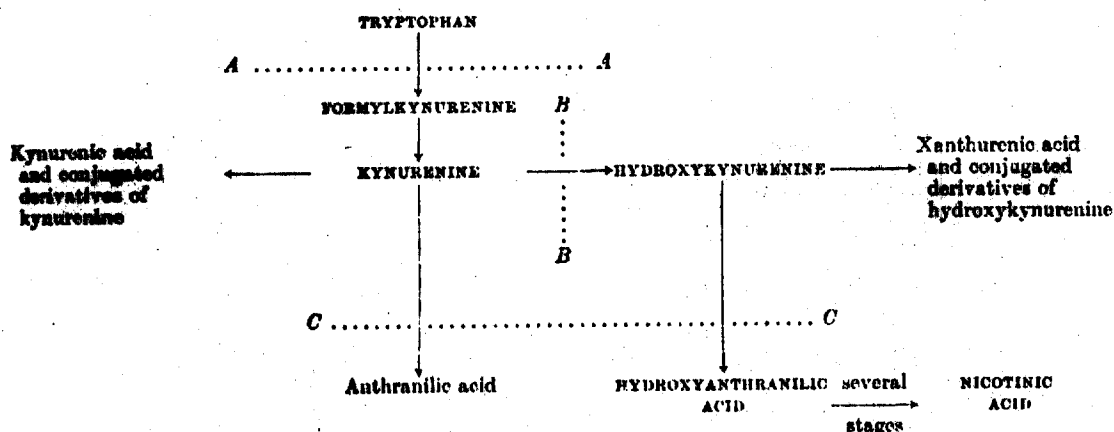


Fig. 4. Outline of the pathway for conversion of tryptophan into nicotinic acid. Intermediates on the main pathway in small capitals, by-products in ordinary type.

These experiments thus establish that thiamine influences tryptophan metabolism at stage *A ... A*, but they do not show whether or not the effect is direct, i.e. whether thiamine is functioning as part of an enzyme cofactor. The reaction involved is quite different in type from the reactions normally considered to be dependent on thiamine. But it is of some interest in this connexion that the two vitamin antagonists oxythiamine and neopyrithiamine have very different actions. Oxythiamine inhibits pyruvate decarboxylation without producing marked neurological changes, whereas neopyrithiamine produces the neurological symptoms without necessarily affecting pyruvate decarboxylation (Woolley & Merrifield, 1954). This strongly suggests participation of thiamine in reactions other than α -keto acid decarboxylation, normally considered to be its prime function.

The inhibition in thiamine deficiency of the first stage of tryptophan metabolism by the kynurenine-nicotinic acid pathway may assist in the

considered unlikely, that biotin is part of a coenzyme which is not readily dissociated from its enzyme in biotin deficiency in the rat. The Indian workers based their results on experiments with a vitamin antagonist, which implies that any such coenzyme is dissociable in *Neurospora*.

SUMMARY

1. The metabolism of tryptophan has been studied in rats deficient in (a) thiamine, (b) thiamine and pyridoxine, (c) biotin, and (d) biotin and pyridoxine.

2. On imposing a thiamine deficiency on pyridoxine-deficient rats, and feeding supplementary tryptophan, the metabolite-excretory pattern typical of pyridoxine deficiency becomes abolished or much reduced. Formation of the substrates of the kynureninase reaction is therefore inhibited. Kynurenine fed at the same stage results in a marked metabolite excretion. No formylkynure-

nine is excreted on giving tryptophan to rats with a simple thiamine deficiency.

3. It is therefore concluded that thiamine is concerned in the conversion of tryptophan into formylkynurenine.

4. Similar experiments combining biotin and pyridoxine deficiencies suggest that biotin is unlikely to be concerned in any stage in the conversion of tryptophan into hydroxykynurenine.

5. Considerable advantages were found in giving vitamins by stomach-tube rather than by mixing them in the diet.

I thank Dr A. M. Copping for her advice, and Mr A. Asatoor and Miss R. Paul for skilled assistance.

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**INSTITUTE OF GENERAL PATHOLOGY OF THE UNIVERSITY
OF CATANIA**

Director, Prof. V. Bisceglie

**RESEARCH ON VITAMIN FACTOR ON EXPERIMENTAL TUMORS
BY 1 : 2 BENZOPYRENE**

**1. The vitamin B₁ (aneutin) action on blastomas
(with a graphic)**

Prof. ALFIO DI GRAZIA

Voluntary Assistant

The rapid development of research on vitamins, aspecially in recent times, had and continues to play a significant role in the field of oncology. Investigators have for some time searched to determine the relationship between tumors and vitamins, but it is since the discovery in identification, isolation and synthesis of vitamin factors in a pure state that research can better define this relationship. If we were to summarize all the literature related to investigations limited vitamin factors to the influence on carcinogenesis and on the general growth and development of tumors, then we do not have to look very far, without coming back to the same research. Namely we refer back to, past research which was essentially accomplished by studying the possibility of occurrence rate, the growth rate, and the development of metastasis of tumors in animals subject to diets deficient in one or another vitamin factor. Thus the negative aspect of the problem was studied; in other words the effect of vitamin failure on tumors, rather than the positive aspect, - vitamin action

on neoplasia was studied. Here it is important to add, that if experiments were not completed with alimentary diets from the point of view of plastic and energy source then it was decided to study not the deficiency effect of this or that vitamin factor on tumors, but to investigate the effect of multiple vitamin deficiencies on the same neoplasias.

On the other side, when our knowledge on the chemical nature of vitamins and on their mechanism was believed to have made a defection action, it is evident that it was not possible to explain the ultimate effect.

To-day these objections are eliminated, for the majority of vitamins whose precise chemical nature and active mechanisms are known.

* * *

In a series of works, I was involved in the effects on various experimental vitamin factors caused by 1 : 2 benzopyrene.

It is timely to define once and for all that vitamins generally are not associated with spontaneous appearances of tumors, but they could eventually effect an already existing tumor in terms of growth speed and in the capacity to cause metastasis, cachexy and so on...

It is quite true, that Japanese authors (SAIKI) discovered the development of neoplasms at the prestomach level in rats, who had been subjected to unbalanced diets of vitamins, however further researches could not confirm this fact. Researches of Bisceglie performed on same terms that only rats held on unbalanced vitamin diets at the prestomach level demonstrated alterations, which could be only interpreted as precancerous lesions, which could potentially develop a cancer, but not necessarily so. Taking into account these concepts, I have proposed to study the action of various vitamin factors on neoplasia caused experimentally.

In this note I refer to results of my researches on the vitamin B (aneurine) effect on tumors of 1 : 2 benzpyrene.

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* *

This study seems important to me for the information we now have on either the action of aneurine mechanism or for kindred on respiratory idleness of the neoplastic cells.



Fig. 1.- I: Duration of latency period between the initial treatment with benzopyrene and the spontaneous development of verrucas and papillomas. - II: Latency period between initial treatment with benzopyrene and sudden growth of cancer. - III: Intercurrent time period of the sudden growth of papillomas on their canceration. - IV: Duration of animal survival in the sudden growth of cancer. Hatched columns: control animals. Black columns: animals treated with B₁ vitamin (aneurine). - Days are indicated by ordinate lines.

Without lingering on the respiratory idleness, on which RONDONI repeatedly insisted that excessive polarized attention is focused on researchers,

I only recall that the tumor cell, (perhaps due to its lability) exploits the energy included in the molecule of glucides only by glycolytic scission; i. e. there is a notion of erobic glycolysis of the tumor cell.

On the other side, it is known that the aneurin is involved in the intermediate metabolism of glucides. Precisely in this intermediate metabolism, after the glycolytic phase, the bodies of 6 atoms of carbon (esosio= ?) are joined to bodies of three atoms of carbon (methylglyoxalic lactic acid) of the aneurin in the cocarboxylase form which is an ester of the pyropho-phoric acid of aneurin and intervenes favoring the oxidation of the pyruvic acid in the acetaldehyde.

Finally the aneurin, evolves into the oxidative phase of intermediate exchange of glucides.

If, however, this form of aneurin is so important for the oxidation of glucides, it is timely to ask: can this vitamin factor increase or facilitate in any way the oxidation of glucides, from the neoplastic cell ? It seems possible to have a glimpse of the interactions between aneurin and tumors. In anyway in addition to these relations, it is interesting to study the effect of a vitamin strait factor (such as aneurin) on tumors experimentally caused by benzopyrene.

Material and technique of invesrigation

For the present research, I used 40 white rats as experimental animals. They were divided into two groups of 20 animals each.

The animals of both groups were subject to three week drainages at the level of loins-sacral region with a benzolic solution of 1 : 2 benzpyrene at 1 %. Thus the animals simultaneously and for entire duration of the experiment received aneurin of a dose of 0.20 mg. The aneurin applied was from

the Firm Bajer (Betaxin). Animals were then retained and the intercurrent latency period was taken into account i.e. between the initial treatment with benzpyrene and the spontaneous appearance of neoplasia, speed of blastoma growth, production of the metastasis and duration of the animal life.

Research results

The course of neoplasia in the control animals, treated with benzpyrene was a typical one, and it is known that after an average latency period of 154 days and after stabilization of the alopecia, there was a sudden growth of verrucca which transmuted into cancer on the average of 34 days. Neoplasias occurrences follow their normal growth, and generally after 43 days of blastoma appearance, the animal died. As a result of these animal experiments, it was determined a certain degree of cachexy which was an evidence of the weight decrease of the same animals. This decrease was approximately 4.8 gr..

In all of control animals I was never able to evaluate the existence of macroscopic metastasis during autoptic examinations.

In view of such behavior in the developing tumors of control animals, the following results have been obtained after treatment with vitamin B₁:

As far as intercurrent latency period is concerned it was ^{on} the average 123 days between the beginning of treatment and sudden appearance of verrucas. This time period is shorter than that of control animals. The cancer of such animals was observed on the average 46.5 days after the appearance of the verrucas. This shows that canceration in animals treated with a B₁ vitamin, occurs in a longer time period than in uncontrolled animals.

For the later cases the intercurrent period between verrucas appearance and papillomas and of cancer beginning was on the average 34 days.

Calculating, however, the latency period between the beginning of treatment with benzpyrene and the appearance of tumors, it was observed that in control animals the time period was on the average 188 days, but in animals treated with vitamin B₁ was on the average 169.5 days, and that animal survival after the appearance of the cancer was 35 days.

The numbers, however, show that if the latency period for sudden appearance of verrucas and papillomas appearance is reduced, then the necessary time period is increased because papillomas are transforming into cancer. This result obtained by me is in agreement with MAISIN, POURBEAIX and CAMERMAN.

The animal survival duration was shorter than that of control animals. Infact, for rats undergoing vitamin B₁ treatment, the survival duration was on the average 207 days, and for control animals was 231 days. The faster elapse of the neoplasia of animals treated with B₁ vitamin does not affect the general condition of the organism; the weight decrease of treated animals was nearly equal to that of the control animals.

It was not possible to evaluate the presence of the metastasis during autoptic examination even on treated animals.

SUMMARY

After having considered the relations between vatamins and tumors and indicated the action mechanism of B₁ vatamin (aneurin), the author relates upon the results of researches he executed on the action of B₁ vitamin upon the developing and course of tumors, which were experimentally obtained with benzopyrene.

After these researches, he found that the B₁ vitamin (aneurine) gets a shortening of the latent period between beginning of the benzopyrene treatment and apparition of warts and papillones, while it gets a prolongation of the time which is necessary for the papilloma cancerization. Moreover, the treatment with B₁ vitamin gets a shortening of the survival of animals.

The cachexy following the neoplasia and the formation of macroscopical metastasis do not seem to be influenced by the aneurine.

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**Ricerche sull'azione dei fattori vitaminici
sui tumori sperimentali da 1:2 benzopirene.**

I. L'azione della vitamina B₁ (ancurina) sui blastomi.

(CON UN GRAFICO)

Prof. ALFIO DI GRAZIA
AIUTO VOLONTARIO

Il rapido svolgersi, specie in questi ultimi tempi delle ricerche sulle vitamine ha avuto e continua naturalmente ad avere il suo riflesso in oncologia. Non sono, come è noto, di oggi, le indagini tendenti a chiarire i rapporti tra tumori e vitamine, ma sono di questi ultimi tempi (dopo la scoperta dei fattori vitaminici allo stato puro e alla realizzazione della loro sintesi) le ricerche che meglio tendono a definire le relazioni tra vitamine e tumori. Se si volesse qui riassumere tutta la letteratura riguardante le indagini seguite per chiarire se e dentro quali limiti i fattori vitaminici hanno influenza non solo per la cancerogenesi ma bensì anche per la crescita e lo sviluppo dei tumori, dovremmo andare molto per le lunghe, ma senza rifarci alla ormai estesa letteratura, è qui opportuno avvertire che in passato tali ricerche essenzialmente si venivano compiendo studiando la possibilità di insorgenza, la velocità di accrescimento, la formazione di metastasi, ecc. di animali sottoposti a regimi alimentari carenzati in questo o quel fattore vitaminico. Si studiava così il lato negativo, per così dire, del problema; l'influenza cioè della mancanza di vitamine su tumori, ma non il lato positivo, l'azione cioè delle vitamine sulle

neoplasie. A ciò bisogna aggiungere poi che se gli esperimenti non erano compiuti con diete alimentari complete dal punto di vista plastico ed energetico si veniva a studiare in definitiva non l'influenza della deficienza di questo o quel fattore vitaminico sui tumori ma bensì si indagavano in definitiva l'influenza di carenze multiple sulle neoplasie stesse.

D'altra parte sino a quando le nostre conoscenze sulla natura chimica delle vitamine e sul loro meccanismo di azione hanno fatto difetto è evidente che non era possibile poter spiegare l'eventuale effetto constatato.

Oggi queste obiezioni sono eliminate in quanto che si può dire che per la maggioranza delle vitamine noi conosciamo la loro precisa natura chimica ed il loro meccanismo di azione.

★ ★

In una serie di lavori io mi sono venuto occupando dell'azione dei diversi fattori vitaminici sui tumori sperimentali provocati da 1:2 benzopirene.

E' qui opportuno definire una volta per sempre che ormai è sicuro che le vitamine in genere non hanno importanza per la insorgenza dei tumori, ma bensì possono eventualmente influenzare un tumore già insorto sia per quel che riguarda la velocità di accrescimento che la capacità di dare metastasi, cachessia e via discorrendo.

E' bensì vero che autori giapponesi (SATO) annunziarono di avere ottenuto a livello del pre-stomaco dei ratti sviluppo di neoplasmi sottoponendo questi animali a diete squilibrate in vitamine, ma ricerche successive non hanno potuto confermare questo fatto. E ricerche di Bisceglie compiute nello stesso senso misero soltanto in rilievo a livello del pre-stomaco dei ratti tenuti a diete squilibrate in vitamine, alterazioni che potevano essere interpretate solo come lesioni precancerose, come lesioni cioè dalle quali può sorgere ma non necessariamente sorge un cancro. Tenendo presente questi concetti io mi sono proposto di studiare l'azione di diversi fattori vitaminici sul decorso di neoplasie sperimentalmente provocate.

Nella presente nota riferisco i risultati delle mie ricerche sulla influenza della vitamina B₁ (aneurina) sui tumori da 1:2 benzopirene.

Un tale studio a me è sembrato importante per le nozioni che oggi si possiedono sia sul meccanismo di azione della aneurina, sia

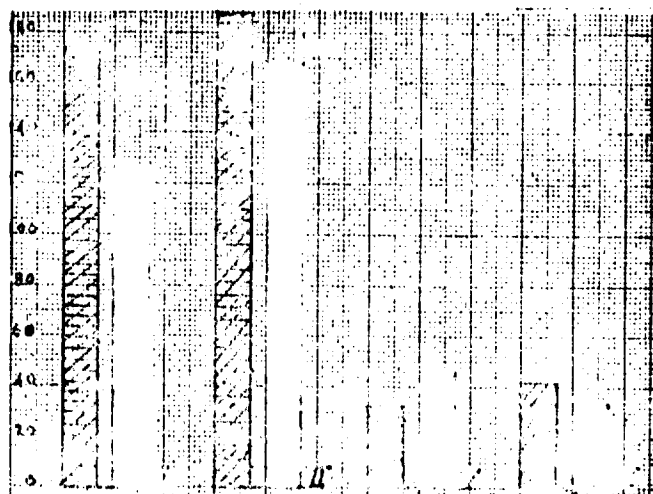


Figura 1. - I: Durata di latenza tra inizio del trattamento con benzopirene ed insorgenza di verruche e papillomi. - II: Periodo di latenza tra inizio del trattamento con benzopirene ed insorgenza del cancro. - III: Periodo di tempo intercorrente dalla insorgenza dei papillomi alla cancerizzazione di questi ultimi. - IV: Durata di sopravvivenza degli animali dalla insorgenza del cancro. - Colonne tratteggiate: animali controllo. - Colonne nere: animali trattati con Vitamina B₁ (aneurina). - Sulla linea delle ordinate i giorni.

per le cognizioni sulla pigrizia respiratoria delle cellule neoplastiche.

Senza fermarmi su tale pigrizia respiratoria, su cui come ripetutamente ha insistito RONDONI si è in maniera eccessiva polarizzata la attenzione degli studiosi, ricordo solo che la cellula tumorale, (forse in grazia della sua labilità) sfrutta l'energia racchiusa nella molecola

dei glucidi solamente per via di scissione glicolitica; si ha cioè la nota glicolisi aerobica della cellula tumorale.

D'altra parte, come è noto, l'aneurina ingrana nel metabolismo intermedio dei glucidi. E precisamente in tale metabolismo intermedio, dopo la fase glicolitica per cui da corpi a 6 atomi di carbonio (esosio) si giunge a corpi a tre atomi di carbonio (acido lattico metilgliossale) l'aneurina sotto forma di cocarbossilasi che è l'estere dell'acido pirofosforico dell'aneurina) interviene favorendo la ossidazione dell'acido piruvico in acetaldeide.

In definitiva quindi l'aneurina, ingrana precisamente nella fase ossidativa del ricambio intermedio dei glucidi.

Se dunque in tal modo l'aneurina ha una sì alta importanza nell'ossidazione dei glucidi è lecito domandarsi: può questo fattore vitaminico incrementare o comunque agevolare l'ossidazione dei glucidi, da parte della cellula neoplastica? Come appare è possibile intravedere interazioni tra aneurina e tumori. In ogni modo a parte tali rapporti or ora prospettati mi è parso interessante studiare l'influenza di un fattore vitaminico puro (quale l'aneurina) sui tumori sperimentalmente provocati con 3,2 benzopirene.

Materiale e tecnica di studio

Per le presenti ricerche mi sono servito come animali da esperimento di numero 40 topi bianchi, i quali sono stati divisi in due lotti di 20 animali ciascuno.

Gli animali di ambedue i lotti sono stati sottoposti a sgocciolamento tri-settimanale a livello della regione lombo-sacrale di soluzione benzolica di 1:2 benzopirene all'1 %. Quindi un lotto di animali ha ricevuto contemporaneamente e per tutta la durata dell'esperimento aneurina nella dose di mg 0,20. L'aneurina adoperata è stata quella della casa Bajer (Betaxin). Gli animali sono stati quindi seguiti e si è tenuto conto del periodo di latenza intercorrente tra inizio del trattamento col benzopirene e l'insorgenza della neoplasia, velocità di accrescimento del blastoma, produzione di metastasi, durata di vita dell'animale.

Risultati delle ricerche

Il decorso delle neoplasie negli animali controllo, trattati cioè con solo benzopirene è stato quello tipico e ben noto e cioè dopo un periodo di latenza in media di 151 giorni e dopo lo stabilirsi della nota alopecia si è avuto insorgenza di verruche che in media dopo 84 giorni si tramutavano in cancro. Le neoplasie insorte seguivano il loro normale accrescimento e in genere dopo 43 giorni dell'insorgenza del bilastoma l'animale veniva a morte. A carico di questi animali era dato di constatare un certo grado di cachessia che tra l'altro era testimoniato dalla diminuzione del peso degli animali stessi. Tale diminuzione infatti, si aggirava in media intorno a g 4,8.

In tutti gli animali controllo all'esame autoptico non mi è stato dato mai apprezzare l'esistenza di metastasi macroscopiche.

Di fronte ad un tale comportamento dei tumori sviluppatosi negli animali controllo, si è avuto negli animali trattati con vitamina B₁ il seguente risultato:

Per quel che riguarda il periodo di latenza intercorrente tra inizio del trattamento ed insorgenza di verruche questo è stato in media di 123 giorni, di un periodo di tempo cioè inferiore a quello degli animali controllo. Il cancro in tali animali si è verificato però dopo giorni 46,5 in media dalla insorgenza di verruche. Ciò dimostra che la cancerizzazione negli animali trattati con vitamina B₁ si realizza in un periodo di tempo maggiore che non nei controlli. In questi ultimi infatti il periodo intercorrente tra insorgenza di verruche e di papillomi e nascita del cancro è stato in media di 31 giorni.

Calcolando però il tempo di latenza tra inizio del trattamento con benzopirene ed insorgenza dei tumori si è constatato che mentre negli animali controllo tale periodo è stato in media di 188 giorni, negli animali trattati invece con vitamina B₁ esso è stato in media di giorni 169,5 e la sopravvivenza degli animali dalla insorgenza del cancro è stata di 35 giorni.

Appare dunque dalle cifre riportate che se il periodo di latenza per l'insorgenza di verruche e papillomi è raccorciato, risulta invece aumentato il periodo di tempo necessario perchè i papillomi si trasfor-

mimo in cancro. Que-ultimo risultato da me conseguito si accorda con quanto hanno visto MAISIN, POURBEMIN e CAMERMAN.

Per quel che riguarda la durata di sopravvivenza degli animali, questa è stata più breve di quella degli animali controllo. Infatti, mentre nei topi sottoposti a trattamento con vitamina B₁ la durata della sopravvivenza è stata 207 giorni in media, negli animali controllo invece essa è stata di 231 giorni. Questo più rapido decorrere della neoplasia negli animali trattati con vitamina B₁ non ha però influito in maniera rilevante sulle condizioni generali dell'organismo tanto che la diminuzione di peso degli animali trattati è stata presso a poco uguale a quella degli animali di controllo.

Anche negli animali trattati non è stato dato apprezzare all'esame autoptico presenza di meta-tasi.

RIASSUNTO

Dopo alcune considerazioni sui rapporti fra vitamine e tumori ed alcuni brevi cenni sul meccanismo di azione della vitamina B₁ (anecurina) vengono riferiti i risultati di ricerche sull'azione della vitamina B₁ sullo sviluppo e decorso dei tumori sperimentalmente provocati con benzopirene.

Da tali ricerche è risultato che la vitamina B₁ (anecurina) accorcia il periodo di latenza decorrente fra inizio del trattamento con benzopirene ed insorgenza di verruche e papillomi mentre aumenta il periodo di tempo necessario per la cancerizzazione dei papillomi. Inoltre il trattamento con vitamina B₁ accorcia la durata di sopravvivenza degli animali.

Lo stato cachettico indotto dalla neoplasia nonché la formazione di meta-tasi macroscopiche non sembra vengono influenzate dalla anecurina.

ZUSAMMENFASSUNG

Nach einigen Beobachtungen ueber die Verhältnisse zwischen Vitamine und Geschwülste, und nach einem kurzen Bericht ueber den Wirkungsmechanismus des B₁-Vitamins (Aneurins), werden die Ergebnisse von Untersuchungen ueber die Wirkung des B₁-Vitamins auf die Entwicklung und den Verlauf der mit Benzopyren experimentell erzeugten Geschwülste angeführt.

Es wurde dabei beobachtet, das, das B₁-Vitamin (Aneurin) die Latenzzeit zwischen dem Beginn der Benzopyrenbehandlung und der

Entstehung von Warzen und Papillomen verkürzt, während es die für die Kancerisation der Papillome nötige Zeit verlängert. Ferner verkürzt die Behandlung mit B₁-Vitamin die Überlebensdauer der Tiere.

Die durch die Neoplasie erzeugte Kachexie und die Bildung makroskopischer Metastasen scheinen vom Aneurin nicht beeinflusst zu werden.

RÉSUMÉ

Après quelques considérations sur les rapports entre vitamines et tumeurs, et après quelques indications sur le mécanisme d'action de la vitamine B₁ (aneurine), l'A. reporte les résultats de recherches qu'il a exécutées sur l'action que la vitamine B₁ a sur le développement et le cours des tumeurs provoquées par voie expérimentelle moyennant le benzopyrène.

Ces recherches ont démontré que la vitamine B₁ (aneurine) réduit la période de latence entre commencement du traitement avec le benzopyrène et apparition de verrues et de papillomes, tandis que elle prolonge le temps nécessaire pour la cancérisation des papillomes; de plus, le traitement moyennant la vitamine B₁ réduit la durée de survie des animaux.

L'état de cachexie provoqué par la néoplasie et la formation de métastases macroscopiques ne semblent pas être influencés par l'aneurine.

SUMMARY

After having considered the relations between vitamins and tumours and indicated the action mechanism of B₁-vitamine (aneurine), the A. relates upon the results of researches he executed on the action of B₁-vitamine upon the developing and course of tumours, which were experimentally got with benzopyrene.

After these researches, he found that the B₁-vitamine (aneurine) gets a shortening of the latent period between beginning of the benzopyrene treatment and apparition of warts and papillomes, while it gets a prolongation of the time which is necessary for the papillome cancerisation. Moreover, the treatment with B₁-vitamine gets a shortening of the survival of animals.

The cachexy following the neoplasia and the formation of macroscopical metastasis do not seem to be influenced by the aneurine.

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Effects of Thiamine on the Growth of Spontaneous Tumors in Mice.

Dobrovolskaia-Zavadskaiia, N.

The oxidation of glucides by normal cells in an organism finally results in the transportation of these glucides into CO_2 and H_2O , which are eliminated by respiration. According to Liebig (1847), Araki (1891) and Zillesen (1891), the normal cells of the body do not secrete lactic acid when oxygen is cut off from them. For example, embryonic tissue is able to live for a certain time in these conditions, deriving its energy from the breakdown of glucose into lactic acid ($\text{C}_6\text{H}_{12}\text{O}_6 = 2\text{C}_3\text{H}_6\text{O}_3$), as the "Mucor mucedo" of Pasteur: fermentation is ceased upon transition from anaerobiosis to aerobiosis. Warburg (1926) objects to the respiration of glycolysis - reaction of the molecular scission of sugar - and finds that the latter took place in all of the varied cancerous cells, not only in anaerobic conditions but equally in aerobic live; the respiration continuing to be an insufficient source of energy.

The primordial role of vitamin B1 in the assimilation and the metabolism of glucides is already known. Help from this product has been interposed on cancer growth studies: Nakahara, Mori and Fujiwara (1939) have tried to prevent the appearance of liver tumors by azolic substances. The result has been questionable: 52.4% of hepatic cancers compared to 50-100% of cancers in the control group. Partial inhibition of cancers to benzopyrene was obtained in a portion of mice treated preventatively with vitamin B1 (28.5% instead of 55.1% in the control series) by Maisin, Pourbaix, and Camerman (1939). No differences were observed by Gordonoff and Ludwig (1938) in the growth of tumors provoked by methyl-cholanthrene in two groups of mice - one put on a diet poor in Vitamin B and the other rich in vitamin B.

The influence of vitamin B1 on the spontaneous growth of tumors was analyzed: 19 mice with 34 mammary adenocarcinomas have been subjected to subcutaneous injections of thiamine (0.2-0.3%), each time freshly prepared. The daily dose of 0.4-0.6 and up to 1 mg. (given to 3 mice for more than 1 month) was tolerated. The tumors were measured periodically in two dimensions and the quantity of growth was calculated by the same method used in an analogous study on vitamin C.

Some details on the results obtained are: the rate of growth, checked before the beginning of treatment for 28 tumors fluctuated between 0.08 and 0.75 giving an average of 0.34 (for total of 181 days of observation). This constant is lowered in the period immediately following the beginning of treatment to 0.12 (individual fluctuations of 0.2 to 0.4). The average calculated on 1000 days of observation in 25 cases and the constant average of 0.23 (140 days of observation) did not reach the before treatment average.

The decrease in the rapidness of growth progressed until it changes into a regression in 15 cases (53.6%). Subsequent increase in the rapidness of growth was only proven in 4 cases with a constant average of 0.15 (for 118 days of observation) and variations of 0.05 and 0.23. Three localizations on two mice developed during the course of treatment. The majority of the mice (13) were treated until their natural death. But the treatment of 6 mice with 13 tumors was interrupted: 6 of these tumors continued their evolution without an acceleration in growth; 4 kept on growing; and 3 new localizations appeared after the discontinuation of treatment. The length of this period without treatment varied for each mouse from 19 to 52 days.

The survival of the treated animals from the moment of the tumor diagnosis until their natural death indicates that: one single mouse died during the course of the first month (24 days); 8 died during the second month (47 days of the average survival time; and 10 (52.7%) died later (64 to 104 days in an average survival time of 86.5 days). If the results of the control series are compared, it would be seen that only about 20% of the animals not treated survived more than 2 months.

Finally, the subcutaneous injections of vitamin B1 to the cancerous mice has clearly decreased the rapidness of growth of their tumors and has increased the average survival period of these animals. In the course of treatment, the stimulus effect on growth is shown to be very little. On the other hand, the interruption of aneurine injections was followed by a recurrence of growth and the appearance of new localizations in half the cases. In spite of the prolonged survival period, the tumors which were progressively getting bigger did not reach, in general, the considerable proportions they often did in the untreated mice. The verifications indicate, perhaps, that vitamin B1 does interfere in the glucidic metabolism of the tumors. The acid aneurine-pyrophosphoric would be, according to Lohmann, identical to the CO-carboxylase.

Compt. Rend. Soc. Biol. 139:494-495. 1945.

**Sur l'effet de l'ascorbine (vitamine B¹) sur la croissance
des tumeurs spontanées chez les souris,**

par N. DOBROVOLSKAIA-ZAVADSKAIA.

L'oxydation des glucides par les cellules normales de l'organisme aboutit finalement à la transformation de ces glucides en CO₂ et H₂O qui sont éliminés par la respiration. Depuis Liebig (1817), Anst (1821), Zillesen (1831), les cellules normales du corps ne survivent de l'acide lactique que lorsqu'on leur apporte l'oxygène; par exemple, le tissu embryonnaire peut vivre un certain temps dans ces conditions, en puisant son énergie dans le dédoublement de glucose en acide lactique ($C_6H_{12}O_6 = 2 C_3H_6O_3$), tout à fait comme le *Mucor mucedo* de Pasteur: tous deux cessent de fermenter après le passage de l'acide lactique à l'acétaldéhyde. Warburg (1926) oppose à la respiration la glycolyse — réaction de la scission moléculaire du sucre — et trouve que cette dernière a lieu dans toutes les cellules cancéreuses variées, non seulement dans les conditions anaérobies, mais également dans la vie aérobie, la respiration comme source d'énergie restant pour elles toujours insuffisante.

On sait le rôle primordial de la vitamine B₁ dans l'assimilation et le métabolisme des glucides. Cela explique qu'on ait tenté d'intervenir à l'aide de ce produit dans le développement des cancers: Nakatani, Mori et Fujiwara (1939), ont essayé d'empêcher l'apparition de tumeurs du foie par les substances azotées, le résultat a été d'autant plus favorable que les cancers hépatiques vis-à-vis de 50 à 100 p. 100 le cancer dans les séries de contrôle; l'inhibition partielle de cancers au benzène a été obtenue dans un lot de souris traitées dans un but prophylactique par la vitamine B₁ (35,5 p. 100 au lieu de 55,1 p. 100 dans la série de contrôle), par Malsin, Pourbaix et Camerman (1939); aucune différence n'a été observée par Gordonoff et Ludwig (1938), en ce qui concerne le développement des tumeurs provoquées par le méthylcholanthrène dans deux groupes de souris l'un mis à un régime pauvre et l'autre à un régime riche en vitamine B₁.

Nous avons essayé l'influence de la vitamine B₁ sur la croissance des tumeurs constituées spontanément: 19 souris, porteuses de 34 cancers de la mamelle, ont été soumises à des injections sous-cutanées d'une solution d'ascorbine synthétique à 0,2-0,3 m. 100, chaque fois fraîchement préparée. La dose quotidienne de 0,1-0,3 et même de 1 mg. (à 3 souris pendant plus d'un mois) a été bien supportée. Les tumeurs ont été mesurées périodiquement dans deux dimensions et la constante

de croissance a fut calculée par la même méthode que celle qui a été utilisée pour un travail analogue avec la vitamine C (1*).

Voici quelques détails sur les résultats obtenus : la constante de croissance a , évaluée avant le début du traitement pour 28 tumeurs, oscillait entre 0,05 et 0,75 suivant le cas, donnant en moyenne 0,34 (sur le total de 131 jours d'observation). Cette constante s'est abaissée, dans la période qui suit immédiatement le début du traitement, à 0,12 (oscillations individuelles de $-0,2$ à $0,6$) — moyenne calculée sur 131 jours d'observation — dans 25 cas (89,1 p. 100). Elle n'a augmenté que pour 3 cas, la constante a moyenne de 0,23 (sur 140 jours d'observation) n'atteignant pas la moyenne d'avant traitement.

La diminution de la rapidité de croissance a progressé en se transformant en une régression (constante a négative, en moyenne $-0,2$ pour 143 jours d'observation) dans 15 cas (50,3 p. 100); l'augmentation ultérieure peut être en stade de diminution de la rapidité de croissance n'a été constatée qu'en 4 cas avec la constante a moyenne de 0,15 (sur le total de 118 jours d'observation) et variations individuelles de a de 0,05 à 0,23. Trois localisations chez 2 Souris (13) ont été développées au cours du traitement. La majorité des Souris (13) ont été traitées jusqu'à leur mort naturelle; mais le traitement de 6 Souris avec 13 tumeurs a été interrompu avant : 6 de ces tumeurs continuaient leur évolution sans accélération de croissance, 1 ont donné une reprise de la croissance et 2 nouvelles localisations ont fait leur apparition après la suppression du traitement. La durée de cette période sans traitement variait pour les différentes Souris de 19 à 52 jours.

La survie de nos animaux traités, à partir du moment du diagnostic de la tumeur jusqu'à leur mort naturelle, se présente ainsi : une seule Souris est morte au cours du premier mois (24 jours); 8 sont mortes au cours du 2^e mois (47 jours de survie en moyenne) et 10 (52,7 p. 100) ont mortes plus tard (de 64 à 164 jours, survie moyenne de 83,5 jours). Si nous rapprochons ces résultats de notre série de contrôle, nous voyons qu'il n'y eut que 20 p. 100 d'animaux non traités environ qui survivaient plus de deux mois.

En définitive, l'administration sous-cutanée de la vitamine B₁ aux Souris cancéreuses a nettement diminué la rapidité de croissance de leurs tumeurs et a augmenté la survie moyenne de ces animaux. Au cours du traitement, l'effet stimulant la croissance ne s'est manifesté que très peu; par contre, l'interruption de l'administration de l'aucurine fut suivie par la reprise de la croissance et l'apparition de nouvelles localisations dans la moitié des cas. Malgré la survie prolongée les tumeurs, tout en croissant progressivement, n'atteignaient en général pas les proportions considérables qu'elles atteignent souvent chez les Souris non traitées. Ces constatations indiquent, nous le pensons, que la vitamine B₁ intervient dans le métabolisme chimiotaxique des tumeurs : l'acide ascorbique-phosphorique serait, suivant Lohmann, identique à la co-carboxylase.

(Institut du radium de l'Université de Paris.)

(1*) C. R. de la Soc. de Biol., 1943, t. 137, p. 689.

THIAMINE CONTENT IN TISSUES, ACTIVITY OF TRANSKETOLASE, AND EXPERIMENTAL TUMORS FOR VARIOUS THIAMINE ALLOWANCES IN THE ORGANISM

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In the literature there is information on phenomena of thiamine deficiency in patients with malignant neoplasms and on attempts to correct this deficiency with a supplementary assignment of thiamine (1-6). In this connection, the question of the effect of different thiamine allowances in the organism on metabolism in tumors and on their growth acquires an essential significance. Unfortunately, the data in the literature mainly concern the second part of the problem, and the metabolic state in tumors for different thiamine allowances in the organism remains incompletely explained. In particular, data are lacking on the effect of the factor indicated on thiamine content and the activity of the thiamine-dependent enzyme transketolase in tumors and other tissues in the host.

Information on the effect of insufficient or excess thiamine input on malignant growth is sparse and contradictory. It is known (7-9) that completely depriving animals of thiamine retards tumor growth. However a similar situation may hardly correspond to clinical conditions. Of great interest by far would be information on tumor growth under subnormal conditions of thiamine allowance, which do not cause severe B₁-avitaminosis.

What concerns supplementary thiamine administration, is, according to the data of some authors, that an excess of thiamine neither affects tumor growth (10-15) nor retards it (8, 15-17, 18). According to the data of other authors, thiamine administration stimulates malignant growth (7, 11, 19-21).

In connection with the insufficient study of the questions indicated, we set ourselves the problem of studying in more detail the effect of different thiamine allowances in experimental animals on thiamine content and the activity of transketolase in tumors and other tumor-bearing animal tissues, as well as on the growth of a transplanted tumor, for which we chose sarcoma 298 in mice. An analogous study was conducted with La hemocytoblastosis (acute leucosis).

Two series of experiments were conducted. In Series 1, the effect of an insufficient and subnormal thiamine allowance was studied; in Series 2, the effect of excess dosages of this vitamin. The experiments were conducted on mice of the C₅₇B₁/6 strain.

In the course of all the experiments, the animals received a synthetic ration of the following composition: casein, washed of vitamins - 18%, cornstarch - 68%, sunflower oil - 10%, salt mixture, ~~as for~~ Jones and Foster (22) - 4%. The fat and water-soluble vitamin content in 1 kg of the ration was vitamin A - 1000 I.U., vitamin D - 1000 I.U., vitamin E - 60 mg, vitamin K (vicasol) - 1 mg, vitamin B₂ - 5 mg, vitamin B₆ - 5 mg, nicotinamide - 15 mg, calcium pantothenate - 30 mg, folic acid - 1 mg, biotin - 0.2 mg, and cholinechloride - 1500 mg.

Thiamine was administered daily to animals intraperitoneally

in a physiological solution. In the first series of experiments, the animals received 0.5 and 1 μ g (insufficient allowance), 5 μ g (subnormal allowance), and 10 μ g (normal allowance) of thiamine per day. In the second series the dosages of thiamine were 10, 100, and 200 μ g per day. In choosing 10 μ g a day as the required dosage of thiamine for mice, we were guided by data from the literature (23-25), as well as by the results of our own preliminary experiments, in which it was established that this dosage guaranteed optimum growth rate in animals with optimum thiamine concentration and transketolase activity in their tissues. The mice were placed on the synthetic diet and received the thiamine dosages indicated above for 17 days, up until the transplantation of sarcoma 298 or La hemocytoblastosis and then during the entire next period of the experiment. On the day of transplantation, part of the animals were killed in order to determine thiamine concentration and transketolase activity in their tissues. The remaining animals were killed after they had developed malignant neoplasms: two-three weeks after sarcoma transplantation and six days after La hemocytoblastosis transplantation.

The percentage of tumor growth inhibition in the animals was the criterion for the effect of different thiamine allowances in sarcoma growth, calculated from tumor weight at the end of the experiment, and the length of life in the animals was the criterion for La hemocytoblastosis.

The over-all thiamine concentration in animal tissues was determined by the modified fluorometric method of Haugen (26, 27).

The weighed portion of the tissue (150-500 mg) was homogenized in 5 ml of 5% trichloroacetic acid (TCA) solution and centrifuged, and the sediment was washed twice in 5% TCA solution. The joint extract, to which 5% TCA solution was added to make a 25-ml volume, was neutralized to a pH of 4.5-5.0 with the addition of 5 ml of 2M sodium acetate solution and was incubated for 15-17 hours at 37 with an extract of the enzyme preparation Aspergillus arizae in order to free the thiamine from its phosphoric esters.

Five ml of the solution obtained was added by pipette to a flask containing 3 ml of freshly prepared potassium ferricyanide solution (4 ml of 1% $K_3Fe(CN)_6$ solution and 15% NaOH solution, to make 100 ml), and was thoroughly mixed for 90 sec. The reaction was ended with the addition of 2 drops of hydrogen peroxide (experimental samples). The control samples were first treated with benzosulfochloride in an alkaline medium (6 drops of 40% NaOH and 1 drop of benzosulfochloride) to destroy the thiamine. Thiochrome was extracted in 10 ml of isobutyl alcohol and its fluoroscein content was determined on an EF-3M fluorometer with an FK-1 first filter and a B₁-2 second filter.

Thiamine content in the tissues was calculated using a calibrated curve constructed for standard thiamine solutions.

Transketolase activity in the animal tissues was determined from the rate of formation of sedoheptulose-7-phosphate (S-7-P) in the incubation of tissue homogenates with ribose-5-phosphate (R-5-P) according to the method of Bruns et al. (28). We modified this method somewhat in relation to diluting the homogenate of the tumor tissue, liver, spleen, and mouse muscle. The optimum homogenate dilution for the tumor was found to be 1:300, for the spleen 1:600, and for skeletal muscle 1:20 using a veronal acetate buffer of pH 7.6. A blood hemolyzate was taken in a 1:10 dilution.

1 ml of tissue homogenate or blood hemolyzate was incubated for 30 and 45 min, respectively, with 0.5 ml of 0.01 M ribose-5-phosphate solution in a 0.01 M veronal acetate buffer solution of pH 7.6. The reaction was ended with the addition of an equal volume of 10% TCA solution. The control solutions were incubated without R-5-P, which was added to the samples after protein sedimentation with TCA. After protein sedimentation by centrifuging, 2 ml of the centrifugate was heated for 80 min with 0.4 ml of concentrated HCl in a boiling water bath. Into the cooled samples was poured 0.4 ml of FeCl_3 in a 2N solution of HCl and 0.2 ml of a freshly prepared solution of orcein in alcohol. The contents of the test tubes were energetically mixed and the samples heated in a boiling water bath again for 3 min. After cooling, 3 ml of 96% alcohol was added to the samples. They were kept in darkness for 4 hr and were photometrically analyzed on an SF-4 at 630 $\text{m}\mu$ against water. Transketolase activity in the tissues was calculated in moles of generating sedoheptulose-7-phosphate in 1 g of tissue in 1 hr, and in blood 1 g of hemoglobin in 1 hr.

In the figure are presented weight curves for the animals which received normal, subnormal, and insufficient thiamine doses. The arrow indicates the day sarcoma 298 was transplanted into them. Data on thiamine concentration and transketolase activity in the tissues of intact animals which received different dosages of thiamine are presented in Table 1 for the day of transplantation of sarcoma 298. As is evident from these data, a state of B_1 -avitaminosis has developed at the time of transplantation of sarcoma 298 in the animals which received insufficient doses of thiamine (0.5 and 1.0 μg a day): their weight began to decrease; transketolase activity in the blood and liver, as well as thiamine concentration in the liver, was significantly decreased. The animals collapsed on the 27th and 30th days of the experiment.

A sharp decrease in thiamine yield in the organism, causing a state of severe B_1 -avitaminosis in the animals, significantly decreased the survival of sarcoma 298 and practically completely suppressed the growth of the transplant tumors (see Table 1). A similar inhibition of malignant tumor growth with thiamine deficiency was noted by other authors (7, 8, 29).

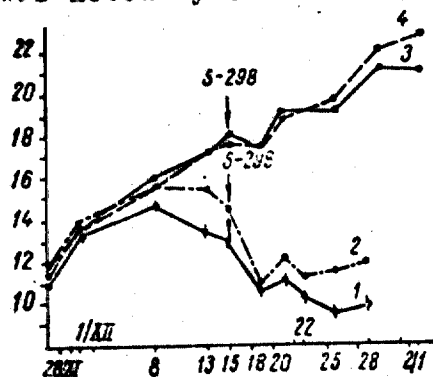


Fig. 1. Weight of animals which received insufficient thiamine doses. Abscissa: day of experiment, ordinate: weight (in g). 1) for 0.5 μg thiamine given to mouse per day; 2) 1 μg ; 3) 5 μg ; 4) 10 μg (physiological requirement). Arrow indicates day of sarcoma 298 transplantation.

In animals which received lower doses of thiamine, transketolase activity in tumor tissues was lowered by 2 times in comparison with transketolase activity in sarcoma of the control animals. The decrease in transketolase activity occurred as well in other tissues of the tumor-bearing animals, in the blood and liver. Thiamine concentration in the liver of these animals was lowered 4.5-7 times, which confirms a state of severe B_1 -avitaminosis (see Table 1). Suppression of malignant growth in similar situations is rather hard to interpret, since this suppression may be caused not only by the release of thiamine-dependent enzymes, but also by such nonspecific factors as malnutrition and worsening of the general

state of the animals, and various secondary disturbances which developed with the B₁-avitaminosis.

Table 1. Effect of Different Thiamine Allowances for the Mouse Organism on Growth of Sarcoma 298 and Biochemical Indices Associated with Thiamine Metabolism and Functions

Index Studied	Thiamine Dose per Mouse per Day (in μ g)			
	0.5 (I)	1 (II)	5 (III)	10 (IV)
Animal weight (in g)	9.7 \pm 0.1	12.0 \pm 0.3	18.3 \pm 0.5	18.8 \pm 0.6
Tumor weight (in g)	0.086 \pm 0.008 $P_{II-I} < 0.001$ $P_{II-I} < 0.05$	0.164 \pm 0.030	3.0 \pm 0.2 $P_{III-II} < 0.001$ $P_{IV-III} < 0.001$	4.6 \pm 0.2
Transketolase activity (in moles of S-7-P for 1 g dried tissue per hr)				
in the tumor	45.0 \pm 7.0 $P_{II-I} < 0.005$	57.0 \pm 5.0 $P_{III-II} > 0.05$	64.0 \pm 4.0 $P_{IV-III} < 0.001$	109.0 \pm 9.0
in the liver	47.0 \pm 2.5	44.2 \pm 2.4	186 \pm 12 $P_{IV-III} > 0.05$	212 \pm 8 74 \pm 7
Transketolase activity in blood (in μ moles of S-7-P for 1 g Hb per hr)	—	—	$P_{IV-III} > 0.05$	
Thiamine concentration (in μ g for 1 g dried tissue)				
in the tumor	—	—	0.4 \pm 0.06	1.2 \pm 0.07
in the liver	0.8 \pm 0.1	0.8 \pm 0.1	3.6 \pm 0.6 $P_{IV-III} > 0.05$	5.6 \pm 0.8

In this connection, of very great interest are the results obtained from the study of sarcoma 298 development under conditions of subnormal thiamine allowance in a 5- μ g dose per mouse per day. The animals which received this dose of thiamine did not starve, their weight did not differ from the weight of the control animals which received an optimum dose of thiamine (10 μ g), and transketolase activity in the blood and liver remained at a normal level. At the same time, thiamine concentration in the tissues of the sarcoma 298 was lowered by 3 times, and transketolase activity by 40%, in comparison with the control animals, which received a physiologically normal dose of thiamine.

This deficiency of thiamine caused an inhibition in the growth of malignant tumors by 35% (see Table 1).

Inhibition of sarcoma 298 growth in animals which received a subnormal dose of thiamine was apparently caused specifically by ~~thiamine deficiency~~ and a decrease in the activity of thiamine-dependent enzymes in the tumor tissue. The data obtained indicate that under conditions of subnormal thiamine allowance in the organism, the tumor suffers from its limited intake to a far greater degree than normal tissues of the tumor-bearing animals and the organism as a whole. This selectivity may be determined by the fact that the tumor grows vigorously and therefore depends much more on thiamine intake than do slowly growing tissues.

In contrast with the solid tumors, L_a hemocytoblastosis (acute leucosis) prove to be practically insensitive to thiamine deficiency. In animals which received 1 μ g of thiamine daily (which corresponds to only 1/10 of their requirements for this vitamin) over 17 days up until leucosis transplantation, the development of hemocytoblastosis proceeded intensively and the length of life was the same as in the control animals (7 days). Thiamine concentration in the liver of these animals was lowered by more than 3 times, and the transketolase activity by 40%, which indicates a state of significant B₁-avitaminosis. In the leucosis animals, transketolase activity in the blood was 2 times higher in the blood of the healthy animals which received the same thiamine dose. This is evidently associated with a sharp increase in the number of immature leucocytes in the blood.

The absence of a thiamine-deficiency effect on the development of L_a hemocytoblastosis apparently indicates some sort of peculiarities in leucosis cell metabolism, which make them more independent of thiamine intake from outside.

In order to answer the question of what sort of effect is exerted on the metabolism and growth of tumors by the introduction into the organism of excess doses of thiamine, which exceed the normal physiological requirements for this vitamin, 100 or 200 μ g of thiamine a day were supplementarily administered to the animals. The control animals received 10 μ g of thiamine.

Biochemical studies indicated that the activity of transketolase and thiamine concentration in the tissues of the animals in the control and experimental groups were normal and did not increase under the influence of the supplementary thiamine dose. Administration of excess doses of thiamine did not exert a substantial influence on the growth of sarcoma 298 in the described series of experiments, in comparison with the animals which received thiamine within the bounds of their physiological requirements. The weight of the tumor in animals which received 100 μ g of thiamine per day was equal to 3.8 + or - 0.2 g, for 200 μ g, 4.3 + or - 0.2 g, and for the control animals 4.6 + or - 0.2 g. Transketolase activity as well as thiamine concentration in the tumors in animals which received excess doses of thiamine did not differ either from these indices in the control animals (Table 2). This confirms that a low thiamine concentration in tumor tissue is not a consequence of insufficient thiamine intake in the animal organisms, but is a characteristic property of tumor tissue, and is entirely adequate to guarantee a high transketolase activity.

The administration of excess doses of thiamine did not exert any sort of influence on the development of L_a hemocytoblastosis either.

The length of time the animals lived in all the groups was the same, 7 days.

Other researchers have observed the absence of an effect of excess thiamine dose on the growth of malignant tumors as well (9-11, 13, 15, 30). However, there are data in the literature on the stimulatory effect of thiamine on the growth of malignant tumors (7, 10-21, 29).

Table 2. Effect of Excess Doses of Thiamine on Sarcoma 298 Growth and Biochemical Indices Associated with Thiamine Metabolism

Index Studied	Thiamine Dose per Mouse per Day (in μg)		
	10 (I) Normal	100 (II) Excess	200 (III) Excess
Animal weight (in g)	18,8 \pm 0,6	18,1 \pm 0,4	18,8 \pm 0,7
Tumor weight (in g)	4,6 \pm 0,2	3,8 \pm 0,2	4,4 \pm 0,2
Transketolase activity in blood (in moles of S-7-P for 1 g Hb per hr)	73,9 \pm 6,9	119 \pm 12	136 \pm 10
	$P_{II-I} < 0,02$		$P_{III-II} < 0,2$
Transketolase activity (in moles of S-7-P for 1 g dried tissue per hr)			
In liver	218 \pm 8	210 \pm 17	193 \pm 16
In tumor	109 \pm 9	117 \pm 8	119 \pm 8
Thiamine concentration (in μg for 1g dried tissue)			
In liver	5,6 \pm 0,8	6,5 \pm 1,1	6,6 \pm 0,8
In tumor	1,20 \pm 0,07	1,4 \pm 0,2	1,7 \pm 0,4
		$P_{III-I} > 0,05$	

The contradictory in the literature data may be associated with the fact that in the majority of investigations, the thiamine allowance of the control animals was not sufficiently accurately determined. In our experiments, the control animals received a physiologically normal amount of thiamine and according to the data from biochemical studies they were fully provided with thiamine. Under these conditions, supplementary doses of thiamine exceeding the physiological requirements by 10-20 times did not affect the growth of sarcoma 298. However, when the animals received a subnormal amount of thiamine, even a relatively small increase in its dosage (from 5 to 10 μg per mouse per day) led to an acceleration in sarcoma 298 growth. In this connection, it is possible to say that the stimulation of malignant tumor growth under the influence of excess thiamine dosages, which has been noted by several authors, may be determined by the fact that the animals in the control group in these experiments did not receive optimum amounts of thiamine, corresponding to their complete physiological requirements. Having been observed in these experiments, the stimulation of tumor growth may be caused by the fulfilling of something of a thiamine deficiency in the organism, and not by the effect of its excess. In interpreting these data, it is difficult to exclude the possibility of the sensitivity of various tumor strains to thiamine.

The possibility of accelerating tumor growth in fulfilling a relative thiamine deficiency present in the organism, the solution must be considered, in our view, to the question of the expediency of prescribing thiamine in an oncological clinic, since there is a series of data which indicates that patients with malignant neoplasms are often found in a state of something of a B₁-hypovitaminosis (1-5, 31-32)

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СОДЕРЖАНИЕ ТИАМИНА В ТКАНЯХ, АКТИВНОСТЬ ТРАНСКЕТОЛАЗЫ И РАЗВИТИЕ ЭКСПЕРИМЕНТАЛЬНЫХ ОПУХОЛЕЙ ПРИ РАЗЛИЧНОЙ ОБЕСПЕЧЕННОСТИ ОРГАНИЗМА ТИАМИНОМ

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При недостаточной обеспеченности мышей тиамином (0,5—1,0 мкг в день при физиологической потребности 10 мкг), вызывающей снижение веса и гибель части животных, а также резкое уменьшение содержания тиамина и активности транскетолазы в тканях, наблюдается значительное уменьшение прививаемости саркомы 298 и почти полное подавление роста привившихся опухолей. При субнормальной обеспеченности мышей тиамином (0,5 мкг в день), когда внешние признаки В₁-авитаминоза отсутствуют, а активность транскетолазы в крови и печени животных не отличается от контроля, рост саркомы 298 замедляется на 35%. Концентрация тиамина в опухолевой ткани снижается при этом в 3 раза, а активность транскетолазы — на 40% по сравнению с контролем (введение 10 мкг тиамина в день). На развитие гемочитобластома La недостаток тиамина влияние не оказывает. Избыточные дозы тиамина, превышающие физиологическую потребность в 10—20 раз, не влияют на содержание тиамина и активность транскетолазы в опухолевой ткани, а также рост саркомы 298 и развитие гемочитобластома La.

В литературе есть сведения о явлениях тиаминовой недостаточности у больных со злокачественными новообразованиями и попытках коррекции этой недостаточности дополнительным назначением тиамина [1—6]. В связи с этим приобретает существенное значение вопрос о влиянии различной обеспеченности организма тиамином на обмен веществ в опухолях и их рост. К сожалению, литературные данные касаются главным образом второй части проблемы, а состояние обмена веществ в опухолях при различной обеспеченности организма тиамином остается не вполне выясненным. В частности, отсутствуют данные о влиянии указанного фактора на содержание тиамина и активность тиаминазависимого фермента транскетолазы в опухолях и других тканях хозяина.

Сведения о влиянии недостаточного или избыточного поступления тиамина на злокачественный рост скудны и противоречивы. Известно [7—9], что полное лишение животных тиамина тормозит рост опухолей, однако подобная ситуация вряд ли может соответствовать клиническим условиям. Гораздо больший интерес представляли бы сведения о росте опухолей в условиях субнормальной обеспеченности тиамином, не вызывающей тяжелого В₁-авитаминоза.

Что касается дополнительного введения тиамина, то, по данным одних авторов, избыток тиамина не влияет на рост опухоли [10—15] или тормозит его [8, 15—17, 18], по данным других авторов, введение тиамина стимулирует злокачественный рост [7, 11, 19—21].

В связи с недостаточной изученностью указанных вопросов мы поставили перед собой задачу более детально исследовать влияние различной обеспеченности организма экспериментальных животных тиамином на содержание тиамина и активность транскетолазы в опухоли и других тканях животных-опухоленосителей, а также на рост переносимой опухоли, в качестве которой нами была выбрана саркома 298 мышей. Аналогичное исследование было проведено с гемочитобластомом La (острым лейкозом).

Проведены 2 серии экспериментов. В I серии изучено влияние недостаточной и субнормальной обеспеченности тиамином, во II — влияние избыточных доз этого витамина. Опыты были выполнены на мышах линии С₅₇BL/6.

В течение всего эксперимента животные получали синтетический рацион следующего состава: казеин, отмытый от витаминов, — 16%, мановой крахмал — 68%, подсолнечное масло — 10%, соевая смесь по Джонсу и Фостеру [22] — 4%. Состав жирорастворимых витаминов на 1 кг рациона: витамин А — 1000 МЕ, витамин D —

1000 ИЕ, витамин Е — 60 мг, витамин К (викасол) — 1 мг, витамин В₂ — 5 мг, витамин В₆ — 5 мг, никотинамид — 15 мг, пантогенат кальция — 30 мг, фолиевая кислота — 1 мг, биотин — 0,2 мг, холинхлорид — 1500 мг.

Тиамин вводили животным ежедневно внутривентрально в физиологическом растворе. В I серии опытов животные получали 0,5 и 1 мкг (недостаточная обеспеченность), 5 мкг (субнормальная обеспеченность) и 10 мкг (нормальная обеспеченность) тиамин в день. Во II серии дозы тиамин составляли 10, 100 и 200 мкг в день. Выбирая в качестве дозы, обеспечивающей потребность мышей в тиамине, 10 мкг в день, мы руководствовались данными литературы [23—25], а также результатами собственных предварительных экспериментов, в которых было установлено, что эта доза обеспечивает оптимальную скорость роста животных при оптимальной концентрации тиамин и активности транскетолазы в их тканях. Мыши находились на синтетическом рационе и получали указанные выше дозы тиамин в течение 17 дней до перевивки саркомы 298 или гемоцитобластома La и затем в течение всего последующего периода опыта. В день перевивки часть животных забивали для определения концентрации тиамин и активности транскетолазы в их тканях. Остальных животных забивали после развития у них злокачественных новообразований: через 2—3 недели после перевивки саркомы и через 6 дней после перевивки гемоцитобластома La.

Критерием влияния различной обеспеченности организма тиамин на рост саркомы служил процент торможения роста опухоли, вычисленный по ее весу в конце опыта; для гемоцитобластома La — продолжительность жизни животных.

Концентрацию общего тиамин в животных тканях определяли модифицированным флюорометрическим методом Хаугена [26, 27].

Навеску ткани (150—500 мг) гомогенизировали в 5 мл 5% раствора трихлоруксусной кислоты (ТХУ), центрифугировали и осадок дважды промывали 5% раствором ТХУ. Объединенный экстракт, доведенный 5% раствором ТХУ до объема 25 мл, нейтрализовали до pH 4,5—5,0 добавлением 5 мл 2 М раствора ацетата натрия, инкубировали в течение 15—17 часов при 37° с экстрактом ферментного препарата *Aspergillus oryzae* для высвобождения тиамин из его фосфорных эфиров.

5 мл полученного раствора добавляли из пипетки в колбу, содержащую 3 мл свежеприготовленного раствора феррицианида калия (4 мл 1% раствора $K_3Fe(CN)_6$ и 15% раствор NaOH до 100 мл) и перемешивали в продолжение 90 сек. Реакцию прекращали добавлением 2 капель перекиси водорода (опытные пробы). Контрольные пробы предварительно обрабатывали бензолсульфохлоридом в щелочной среде (6 капель 40% NaOH и 1 капля бензолсульфохлорида) для разрушения тиамин. Тioxром экстрагировали 10 мл изобутилового спирта и его флюоресценцию определяли на флюорометре ЭФ-3М с первичным фильтром ФК-1 и вторичными В₁-2.

Содержание тиамин в ткани рассчитывали с помощью калибровочной кривой, построенной на стандартных растворах тиамин.

Активность транскетолазы в животных тканях определяли по скорости образования седогептулозо-7-фосфата (С-7-Ф) при инкубации гомогенатов тканей с рибозо-5-фосфатом (Р-5-Ф) по методу Bruns и соавт. [28]. Нами была проведена некоторая модификация этого метода в отношении разведения гомогената опухолевой ткани, печени, селезенки и мышц мышей. Было найдено оптимальным разведение гомогената опухоли и печени 1:300, селезенки 1:600 и скелетных мышц 1:20 при использовании веронат-ацетатного буфера pH 7,6. Гемолизат крови брали в разведении 1:10.

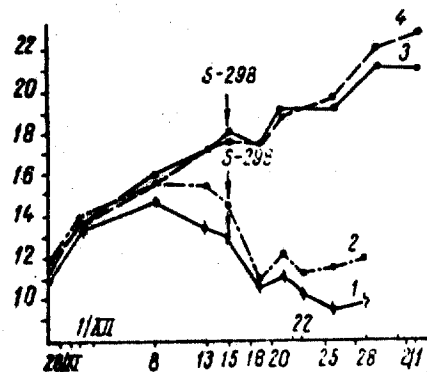
1 мл гомогената ткани или гемолизата крови инкубировали в течение 30 или 45 мин. соответственно с 0,5 мл 0,01 М раствора рибозо-5-фосфата в 0,01 М растворе веронат-ацетатного буфера, pH 7,6. Реакцию прекращали добавлением равного объема 10% раствора ТХУ. Контрольные пробы инкубировали без Р-5-Ф, который добавляли к пробам после осаждения белков ТХУ. После осаждения белков центрифугированием 2 мл центрифугата нагревали в течение 80 мин. с 0,4 мл концентрированной HCl на кипящей водяной бане. В охлажденные пробы приливали по 0,4 мл $FeCl_3$ в 2 мл растворе HCl и 0,2 мл 6% свежеприготовленного раствора ортина в спирте. Содержимое пробирок энергично перемешивали и пробы нагревали на кипящей водяной бане еще в продолжение 3 мин. После охлаждения к пробам добавляли по 3 мл 10% раствора NaOH и держивали в продолжение 4 часов в темноте и фотометрировали на СФ-1 при 680 мμ против воды. Активность транскетолазы в тканях рассчитывали в микромолях образующегося седогептулозо-7-фосфата на 1 г ткани в 1 час, а в крови — на 1 г гемоглобина в 1 час.

На рисунке приведены кривые веса животных, получавших нормальные, субнормальные и недостаточные дозы тиамин. Стрелкой указан день перевивки им саркомы 298. Данные о концентрации тиамин и активности транскетолазы в ткани интактных животных, получавших различные дозы тиамин, в день прививки саркомы 298 представлены в табл. 1. Как видно из этих данных, у животных, получавших недостаточные дозы тиамин (по 0,5 и 1,0 мкг в день) к моменту перевивки саркомы 298 развилось В₁-авитаминозное состояние: вес их начинал снижать-

ся, активность транскетолазы в крови и печени, а также концентрация тиамина в печени были значительно снижены. Животные пали на 27—30-й день опыта.

Резкое снижение доставки в организм тиамина, вызывающее у животных состояние глубокого В₁-авитаминоза, значительно снижало прививаемость саркомы 298 и практически полностью подавляло рост привившихся опухолей (см. табл. 1). Сходное торможение роста злокачественных опухолей при недостатке тиамина наблюдали другие авторы [7, 8, 29].

У животных, получавших низкие дозы тиамина, активность транскетолазы в ткани опухоли была снижена в 2 раза по сравнению с активностью транскетолазы в саркоме контрольных животных. Снижение активности транскетолазы происходило и в других тканях животных-опухоленосителей: в крови и печени. Концентрация тиамина в печени этих животных была снижена в 4,5—7 раз, что свидетельствует о состоянии глубокого В₁-авитаминоза (см. табл. 1). Подавление злокачественного роста в подобных условиях довольно трудно интерпретировать, по-



Вес животных, получавших недостаточные дозы тиамина.

По оси абсцисс — дни эксперимента, по оси ординат — вес животных (в г).

1 — по 0,5 мкг тиамина на мышь в день; 2 — по 1 мкг; 3 — по 5 мкг; 4 — по 10 мкг тиамина на мышь в день (физиологическая потребность).

Стрелкой показан день перевивки саркомы 298.

скольку это подавление могло быть обусловлено не только исключением тиаминзависимых ферментов, но и такими неспецифическими факторами, как голодание и ухудшение общего состояния животных, а также различными вторичными нарушениями, развившимися при В₁-авитаминозе.

Таблица 1

Влияние различной обеспеченности организма мышей тиамином на рост саркомы 298 и биохимические показатели, связанные с обменом и функцией тиамина

Исследуемый показатель	Доза тиамина на мышь в день (мкг)			
	0,5 (I)	1 (II)	5 (III)	10 (IV)
Вес животных (в г)	9,7±0,1 $P_{II-I} < 0,001$	12,0±0,3	18,3±0,5 $P_{III-II} < 0,001$	18,8±0,6 $P_{IV-III} < 0,001$
» опухоли (в г)	0,086±0,008 $P_{II-I} < 0,05$	0,164±0,030	3,0±0,2 $P_{IV-III} < 0,001$	4,6±0,2
Активность транскетолазы (в мкмольх С-7-Ф на 1 г сырой ткани в час) в опухоли	45,0±7,0 $P_{II-I} < 0,005$	57,0±5,0 $P_{III-II} > 0,05$	64,0±4,0 $P_{IV-III} < 0,001$	109,0±9,0 $P_{IV-III} > 0,05$
» в печени	47,0±2,5	44,2±2,4	186±12 58±5	212±8 74±7
Активность транскетолазы в крови (в мкмольх С-7-Ф на 1 г Нв в час)	—	—	$P_{IV-III} > 0,05$	
Концентрация тиамина (в мкг на 1 г сырой ткани) в опухоли	—	—	0,4±0,05	1,2±0,07
» в печени	0,8±0,1	0,8±0,1	3,6±0,6 $P_{IV-III} > 0,05$	5,6±0,8

В связи с этим значительно больший интерес представляют результаты, полученные при изучении развития саркомы 298 в условиях субнормальной обеспеченности тиаминном в дозе 5 мкг на мышь в день. Животные, получавшие эту дозу тиамина, не голодали, их вес не отличался от веса контрольных животных, получавших оптимальную дозу тиамина (10 мкг), а активность транскетолазы в крови и печени оставалась на нормальном уровне. В то же время концентрация тиамина в ткани саркомы 298 была снижена в 3 раза, а активность транскетолазы — на 40% по сравнению с контрольными животными, получавшими физиологически нормальную дозу тиамина.

Этот недостаток тиамина вызвал торможение роста злокачественной опухоли на 35% (см. табл. 1).

Торможение роста саркомы 298 у животных, получавших субнормальную дозу тиамина, по-видимому, специфически обусловлено недостатком тиамина и снижением активности тиаминзависимых ферментов в опухолевой ткани. Полученные данные показывают, что в условиях субнормальной обеспеченности организма тиаминном опухоль страдает от его ограниченного поступления в гораздо большей степени, чем нормальные ткани животного-опухоленосителя и организм в целом. Эта избирательность может быть обусловлена тем, что опухоль бурно растет и поэтому гораздо больше зависит от поступления тиамина, чем медленно растущие ткани.

В противоположность солидным опухолям гемоцитобластоз La (острый лейкоз) оказался практически нечувствительным к недостатку тиамина. У животных, получавших до перевивки лейкоза в течение 17 дней по 1 мкг тиамина в день (что соответствует лишь одной десятой их потребности в этом витамине), развитие гемоцитобластоза шло интенсивно и продолжительность жизни была такой же, как и у контрольных животных (7 дней). Концентрация тиамина в печени этих животных была снижена более чем в 3 раза, а активность транскетолазы в ней — на 40%, что указывает на состояние значительного В₁-авитаминоза. У лейкозных животных активность транскетолазы крови была в 2 раза выше активности транскетолазы крови здоровых животных, получавших те же дозы тиамина, что, очевидно, связано с резким увеличением числа незрелых лейкоцитов в крови.

Отсутствие влияния недостатка тиамина на развитие гемоцитобластоза La, по-видимому, указывает на какие-то особенности обмена лейкозных клеток, делающих их более независимыми от поступления тиамина извне.

Для выяснения вопроса о том, какой эффект оказывает на обмен и рост опухоли введение в организм избыточных доз тиамина, превышающих нормальную физиологическую потребность в этом витамине, животным вводили дополнительно по 100 или 200 мкг тиамина в день. Контрольные животные получали по 10 мкг тиамина.

Биохимические исследования показали, что активность транскетолазы и концентрация тиамина в ткани животных как контрольной, так и опытных групп были нормальными и не повышались под влиянием дополнительных доз тиамина. Введение избыточных доз тиамина не оказало существенного влияния на рост саркомы 298 в описанных сериях экспериментов по сравнению с животными, получавшими тиамин в пределах их физиологической потребности. Вес опухоли у животных, получавших 100 мкг тиамина в день, был равен $3,8 \pm 0,2$ г; 200 мкг — $4,1 \pm 0,2$ г; у контрольных животных — $4,6 \pm 0,2$ г. Активность транскетолазы, а также концентрация тиамина в опухоли животных, получавших избыточные дозы тиамина, также не отличались от этих показателей у контрольных животных (табл. 2). Это свидетельствует о том, что низкая концентрация тиамина в опухолевой ткани — это не следствие недостаточного поступления тиамина в организм животных, а характерное свойст-

во опухолевой ткани, и вполне достаточна для обеспечения высокой активности транскетолазы.

Введение избыточных доз тиаминна не оказало какого-либо действия также на развитие гемоцитобластоза La.

Продолжительность жизни животных во всех группах была одинаковой и составляла 7 дней.

Отсутствие влияния избыточных доз тиаминна на рост злокачественных опухолей наблюдали также другие исследователи [9—11, 13, 15, 30]. Однако наряду с этим в литературе имеются данные о стимулирующем влиянии тиаминна на рост злокачественных опухолей [7, 19—21, 29].

Таблица 2

Влияние избыточных доз тиаминна на рост саркомы 298 и биохимические показатели, связанные с обменом тиаминна

Исследуемый показатель	Доза тиаминна на мышь в день (в мкг)		
	нормальная	избыточные	
	10 (I)	100 (II)	200 (III)
Вес животных (в г)	18,8±0,6	18,1±0,4	18,8±0,7
» опухоли (в г)	4,6±0,2	3,8±0,2	4,4±0,2
Активность транскетолазы крови (в мкмольх С-7-Ф на 1 г Нв в час)	73,9±6,9	119±12	136±10
Активность транскетолазы (в мкмольх С-7-Ф на 1 г сырой ткани в час)	$P_{II-I} < 0,02$		$P_{III-II} < 0,2$
» в печени	218±8	210±17	193±16
» в опухоли	109±9	117±8	119±8
Концентрация тиаминна (в мкг на 1 г сырой ткани)			
» в печени	5,6±0,8	6,5±1,1	6,6±0,8
» в опухоли	1,20±0,07	1,4±0,2	1,7±0,4
		$P_{III-I} > 0,05$	

Противоречивость данных литературы может быть связана с тем, что в большинстве исследований не была достаточно точно определена обеспеченность тиаминном контрольных животных. В наших опытах контрольные животные получали физиологически нормальное количество тиаминна и по данным биохимических исследований были полностью обеспечены тиаминном. В этих условиях дополнительные дозы тиаминна, превышающие физиологическую потребность в 10—20 раз, не влияли на рост саркомы 298. В то же время когда животные получали субнормальное количество тиаминна, даже относительно небольшое повышение его дозы (с 5 до 10 мкг на мышь в день) приводило к ускорению роста саркомы 298. В связи с этим можно высказать предположение, что стимуляция роста злокачественных опухолей под действием избыточных доз тиаминна, отмеченная некоторыми авторами, могла быть обусловлена тем, что животные контрольной группы в этих экспериментах не получали оптимальных количеств тиаминна, соответствующих их полной физиологической потребности. Наблюдавшаяся в этих опытах стимуляция роста опухоли могла быть обусловлена восполнением некоторого недостатка тиаминна в организме, а не действием его избытка. При трактовке этих данных трудно исключить и возможность различной чувствительности разных штаммов опухолей к тиамину.

Возможность ускорения роста опухоли при восполнении имеющегося в организме относительного недостатка тиаминна следует, на наш взгляд, учитывать при решении вопроса о целесообразности назначения

тиамина в онкологической клинике, поскольку имеется ряд данных, показывающих, что больные со злокачественными новообразованиями часто находятся в состоянии некоторого В₁-гиповитаминоза [1—5, 31—33].

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L. V. Diyachkova, E. M. Shamaeva, G. N. Platonova, V. B. Spirichev. — THIAMINE CONTENT IN THE TISSUES. ACTIVITY OF TRANSKETOLASE AND DEVELOPMENT OF EXPERIMENTAL TUMOURS WITH DIFFERENT THIAMINE ALLOWANCES IN THE ORGANISM

Summary. With insufficient thiamine allowances in mice (0.5—1.0 γa day and physiological requirement of 10γ) provoking loss of weight and death of some of the animals, along with a drastic diminution of the thiamine content and transketolase activity in the tissues, one can observe a significantly lessened take of sarcoma 298 and a nearly complete suppression of the growth of the already adapted tumours. With subnormal supply of thiamine to the mice (0.5γ a day), when external signs of the B₁ vitamin deficiency are absent and the blood and liver transketolase activity in the animals does not differ from that in controls — the sarcoma 298 growth slackens by 35%. The thiamine concentration in the neoplastic tissue then becomes curtailed to but one third and the transketolase activity falls by 40% in comparison to controls which received 10γ of thiamine per day each. The shortage of thiamine does not affect the development of the La hemocytoblastosis. Exuberant doses of thiamine, exceeding the physiological demand by as many as 10 to 20 times, have no influence on the thiamine content and transketolase activity in the neoplastic tissue, nor do they affect the growth of sarcoma 298 and development of the La hemocytoblastosis.

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9270

Action of Synthetic Vitamin B₁.

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The antineuritic vitamin, B₁, has been crystallized by different groups of investigators,¹⁻⁶ but it remained for Williams and his associates⁷ to arrive at a correct structural formula, confirmed by successful synthesis.⁸

For some time, the crystalline vitamin B₁, both the natural and the synthetic, has been available in the form of the hydrochloride in our

¹ Jansen, B. C. P., and Donath, W. F., *Geneesk. tijdschr. v. Nederl. Indie*, 1927, **66**, 810.

² Odake, S., *Proc. Imp. Acad. Tokyo*, 1931, **7**, 102.

³ Windaus, A., Tschesche, R., Ruhkoff, H., Laquer, F., and Schultz, F., *Z. f. physiol. Chem.*, 1932, **204**, 123.

⁴ Seidell, A., and Smith, M. I., *J. Am. Chem. Soc.*, 1933, **55**, 3380.

⁵ Kinnorsley, H. W., O'Brien, J. R., and Peters, R. A., *Biochem. J.*, 1933, **27**, 232.

⁶ Williams, R. R., Waterman, R. E., and Keresztesy, J. C., *J. Am. Chem. Soc.*, 1934, **56**, 1187.

⁷ Williams, R. R., *J. Am. Chem. Soc.*, 1935, **57**, 229; 1936, **58**, 1063.

⁸ Williams, R. R., and Cline, J. K., *J. Am. Chem. Soc.*, 1936, **58**, 1504; 1937, **59**, 216.

laboratory for pharmacological studies. Our results appear so uniform that they can probably serve as an additional proof to that established by chemical means, concerning the identity of the two products.

To determine the potency of the synthetic vitamin B₁, rats were made definitely polynuritic and treated with various doses according to the technique of Smith,⁹ except that a small amount (0.4%) of Brewers' yeast was added to the diet, as suggested by Dann,¹⁰ in order to reduce the incidence of deaths. Tests were also carried out in pigeons rendered polynuritic by the diet proposed by Cowgill¹¹ for confirmative purposes.

TABLE I.
Potency of Synthetic Vitamin B₁.

Species Used	No. of Polynuritic Animals Used	Dose mg.	No. of Animals Cured
Rats	3	.003	1
	7	.004	6
	13	.005	10
	5	.006	4
Pigeons	9	.005	9

As shown in Table I, doses of 0.004 to 0.006 mg. of synthetic vitamin given by mouth were sufficient to cure 77 to 85% of the rats. An amount of 0.003 mg. was effective in one case but failed in the other 2, so that the minimal curative dose of the vitamin by mouth in rats was 0.004 mg. in the present series of experiments. In 9 pigeons, a dose of 0.005 mg. injected intravenously abolished the symptoms of polynuritis in 4 animals for 2 to 3 days, and in 5 for 4 days or more. The figures in this investigation with both rats and pigeons correspond closely to those reported by other workers for the natural crystalline vitamin,^{6, 12-14} and fully confirm those claimed by Williams and Cline⁸ for their synthetic product.

Other pharmacological effects are also similar when the natural and the synthetic compounds are compared side by side. In an etherized cat weighing 2.63 kg., doses of 5, 20, and 50 mg. of the synthetic product, injected intravenously, caused a slight decrease

⁹ Smith, M. I., *Pub. Health Rep.*, 1930, **43**, 116.

¹⁰ Dann, F. P., *J. Nutrition*, 1930, **12**, 461.

¹¹ Cowgill, G. E., *The Vitamin B Requirement of Man*, Yale Press, New Haven, 1934, 29.

¹² Jansen, B. C. P., Kinnersley, H. W., Peters, R. A., and Reader, V., *Biochem. J.*, 1930, **24**, 1824.

¹³ Ammerman, M., and Waterman, R. E., *J. Nutrition*, 1935, **10**, 25.

¹⁴ Waterman, R. E., and Ammerman, M., *J. Nutrition*, 1935, **10**, 161.

in respiratory volume, but no changes in respiratory rate, blood pressure, or heart rate. Similar effects were observed with the same doses of the natural vitamin. Neither substance produced congestion or necrosis in the rabbit's ear by subcutaneous injection in the dosage of 1 mg. dissolved in 0.1 cc. of saline. The minimal lethal dose (M.L.D.) in guinea pigs is the same with both the natural and the synthetic products, by intravenous injection, as shown in Table II. The vitamin solution employed for the toxicological study was 2% in each case, and the weight of the animals varied from 210 to 265 gm. Clonic convulsions occurred after doses of 150 mg. per kg., or more, had been administered. Those animals which survived the sublethal doses recovered completely within 1½ and 5 minutes, apparently without any after effects.

TABLE II.
Toxicity of Natural and Synthetic Vitamin B₁ Hydrochloride.

Vitamin B ₁ .HCl	Dose mg. per kg.	No. of Pigs Died Over No. Used	M. L. D. mg. per kg.
Synthetic	300	1/1	180
	200	1/1	
	180	2/3	
	160	0/2	
	150	0/1	
	100	0/1	
Natural	180	2/2	180
	160	1/3	

Summary. Results obtained in animals indicate that the natural crystalline vitamin B₁ and the synthetic product are identical.

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THE EFFECT OF VITAMINS ON ACID FORMATION IN SALIVA¹

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In 1936 (1) it was suggested that the mechanism by which acids are formed in the mouth may be analogous to the series of chemical reactions that take place during the metabolism of carbohydrates in muscle tissue and in fermentation by various microorganisms (2, 3, 4, 5, 6, 7, 8). Since that time it has been shown that the above suggestion is true (9). Furthermore, it has been shown that certain chemical compounds that are known to inhibit these reactions also inhibit dental caries. More recently it has been demonstrated that the vitamins, particularly those of the B complex, play a very important role in the metabolism of carbohydrates (10). If this is true, it would furnish a theoretical basis for the various clinical reports concerning the effects of vitamins in the development of dental caries.

It has been reported by Kniesner (11) that people suffering from a vitamin B deficiency have very little caries. Other clinical reports indicate that vitamins A, D, and C may influence caries activity (12, 13, 14, 15, 16). On the basis of these reports it was thought that a study of the effect of these vitamins on the rate of acid formation in saliva would be of value.

EXPERIMENTAL

In view of the difficulties involved in producing and estimating acid formation in saliva, the acid formed was determined by its solution effect on human enamel. This eliminates the difficulties encountered by both the titration method and the measurement of pH, and produces conditions more like those which occur in the mouth. As the acids are neutralized by the human enamel as fast as they are formed, there is no drastic increase in the acidity, which would materially inhibit the acid formation by the normal oral bacteria. Furthermore, it furnishes an indication of the acids formed in excess of the normal buffering capacity of the saliva.

Saliva was gathered by paraffin stimulation, well mixed, and divided into several different portions of 10 cc. each. One sample of the fresh saliva was analyzed for calcium. To each of the other samples was added 1 gm. of glucose and 0.1 gm. of 300 mesh powdered human tooth enamel. In addition to this, appropriate amounts of the test material were added to the samples, with the exception of 2 that were used as controls. Two samples of each concentration were made up in order to secure checks. The samples were then sealed and

¹ This work was done under a grant from the Good Teeth Council for Children. Received for publication December 8, 1943.

placed in a water bath and incubated at 37° C. with constant agitation. This constant shaking is essential in order to facilitate the solution of the enamel as fast as the acids are formed. After 4 hours of incubation the samples were removed and analyzed for calcium. This procedure is essentially the same as the chemical test for caries susceptibility (17). The acids formed were expressed as milligrams of calcium dissolved per 100 cc. of saliva.

The effects of beta carotene (vitamin A); thiamin chloride hydrochloride (vitamin B₁); riboflavin (vitamin B₂); pyridoxine (vitamin B₆); pantothenic acid; nicotinic acid; nicotinic amide; para aminobenzoic acid; ascorbic acid (vitamin C); calciferol (vitamin D); alpha tocopherol (vitamin E); 2-methyl-1,4-naphthoquinone; vitamin K₁; and cholesterol were studied. In addition to the above, "Cerophyl," a commercial product, prepared from grass and containing the grass juice factor, was studied.

RESULTS

The results are shown in Table I. It should be noted that the calcium content of the fresh saliva indicates the condition with no acid formed. The results of the control sample indicates the acid formed during the incubation period when no test material is present. Any material deviation from this figure with the use of the test material indicates either a stimulation or inhibition of acid formation. As can be seen from the table, it is evident that under the conditions of the experiment only one of the vitamins, vitamin K, produced a drastic decrease in the rate of acid formation. This compound in a concentration of 1.3 mg./100 cc. caused a complete inhibition of acid formation. There was some evidence that thiamin, nicotinic acid, "Cerophyl" and cholesterol may cause a slight stimulation of acid production.

DISCUSSION

In considering the above results, one should bear in mind the probable mechanisms involved in the production of acids. For this reason a definite stimulation or a definite inhibition indicates a specific effect and should be a criterion of its effect on dental caries. On the other hand, if no effect was observed, it does not mean that the vitamin would have no effect on caries. It is well known that the role of vitamins in carbohydrate metabolism is exceedingly complicated, and that combinations of vitamins with other vitamins or with other compounds or ions may be necessary to cause a stimulation.

It is interesting to note that although checks were made using the saliva from at least 2 individuals with each vitamin, the results of certain of the vitamins varied somewhat from individual to individual. This was particularly true with the members of the B complex and with cholesterol. Thus, it may well be that in those cases in which no effect was observed, or in which variable results were obtained, a stimulation could be produced, provided the proper combinations could be found, or providing a deficiency of any of the vitamins existed. This is demonstrated by the fact that although none of the individual members of the "B complex" caused a marked stimulation at all concentrations, "Cero-

TABLE I
Effect of various vitamins on acid production

TUBE	AMOUNT ADDED		CALCIUM
	mg./100 cc.	mM./L.	mg./100 cc.
β-Carotene			
Fresh saliva			5.2
1. Control	0	0	24.8
2.	12.5	0.23	23.2
3.	24.0	0.46	21.5
4.	50.0	0.92	20.0
5.	100.0	1.84	22.0
6.	200.0	3.68	20.3
Thiamin			
Fresh saliva			4.5
1. Control	0	0	13.9
2.	14	0.58	19.5
3.	28	1.16	15.6
4.	56	2.32	12.5
5.	112	4.64	13.7
6.	224	9.28	14.7
7.	448	18.56	16.6
Riboflavin			
Fresh saliva			5.2
1. Control	0	0	15.5
2.	14	0.37	15.7
3.	28	0.74	16.5
4.	56	1.48	16.7
5.	112	2.96	16.8
6.	224	5.92	15.9
7.	448	11.84	16.0
Pyridoxine			
Fresh saliva			5.2
1. Control	0	0	24.8
2.	12.5	0.74	21.0
3.	25.0	1.48	18.5
4.	50.0	2.96	20.0
5.	100.0	5.92	20.0
6.	200.0	11.84	18.5
Nicotinic acid			
Fresh saliva			4.8
1. Control	0	0	18.7
2.	14	1.13	24.3
3.	28	2.26	19.4
4.	56	4.52	17.8
5.	112	9.04	18.0
6.	224	18.08	13.2
7.	448	36.16	22.4

TABLE I—Continued

TUBE	AMOUNT ADDED		CALCIUM
	mg./100 cc.	mM./L.	mg./100 cc.
Nicotinic amide			
Fresh saliva			4.3
1. Control	0	0	17.2
2.	14	1.14	17.1
3.	28	2.28	16.5
4.	56	4.56	16.0
5.	112	9.12	22.6
6.	224	18.24	17.3
7.	448	36.48	15.2
p-Aminobenzoic acid			
Fresh saliva			7.5
1. Control	0	0	23.5
2.	10	0.73	21.5
3.	20	1.46	21.0
4.	40	2.92	18.5
5.	80	5.84	18.5
6.	120	8.76	17.5
7.	160	11.68	17.8
8.	200	14.60	16.5
Calcium pantothenate			
Fresh saliva			7.5
1. Control	0	0	23.5
2.	10	0.19	23.5
3.	20	0.38	22.0
4.	40	0.77	21.5
5.	80	1.55	19.0
6.	120	2.33	19.0
7.	160	3.10	19.0
8.	200	3.88	18.0
Ascorbic acid			
Fresh saliva			5.3
1. Control	0	0	24.8
2.	12.5	0.71	21.5
3.	25.0	1.42	21.0
4.	50.0	2.84	21.5
5.	100	5.68	21.5
6.	200.0	11.36	21.5
Vitosterol			
Fresh saliva			5.3
1. Control	0	0	24.8
2.	12.5	0.32	21.8
3.	25.0	0.64	20.7
4.	50.0	1.28	17.5
5.	100.0	2.56	18.5
6.	200	5.12	24.0

TABLE I—Continued

TUBE	AMOUNT ADDED		CALCIUM
	mg./100 cc.	mM./L.	mg./100 cc.
α-Tocopherol			
Fresh saliva			5.3
1. Control	0	0	24.8
2.	12.5	0.29	22.5
3.	25.0	0.58	22.0
4.	50.0	1.16	21.2
5.	100.0	2.32	16.0
6.	200.0	4.64	22.5
2-Methyl-1,4-naphthoquinone			
Fresh saliva			5.6
1. Control	0	0	26.6
2.	1.35	0.078	4.8
3.	2.30	0.133	4.7
4.	3.50	0.203	2.6
5.	8.00	0.465	2.5
6.	11.30	0.658	2.6
7.	18.50	1.075	2.4
Vitamin K₁			
Fresh saliva			5.5
1. Control	0	0	25.0
2.	500	11.12	11.0
Cerophyl			
Fresh saliva			
1. Control	0		23.2
2.	10		27.5
3.	50		29.0
4.	100		37.5
Cholesterol			
Fresh saliva			5.8
1. Control	0	0	10.0
2.	25	0.64	14.0
3.	50	1.29	17.5
4.	125	3.24	18.0
5.	250	6.48	18.0
6.	500	12.96	18.0

phyl," which is composed of all known members of the "B complex" group (including the grass juice factor) and vitamins A and C, caused a significant stimulation of acid formation. This may explain the results of Kniesner, Mann and Spies, who found a decreased caries activity in patients suffering from a vitamin B deficiency, but could not find a significant difference in the salivary concentration of certain members of this group of vitamins. Although it is probable that these results on individual vitamins may not indicate stimulating effects of certain combinations, it is also possible that combinations may produce inhibiting effects. This is extremely unlikely, however, as all of the known inhibitors at present are single compounds.

One should also keep in mind that the saliva used in these experiments was taken from dental students who were in good health but who were caries active individuals. The diet of these individuals was adequate, and hence it would be expected that normal amounts of all of the vitamins would be present in the saliva. Insofar as the vitamins are usually active in very small quantities, it is quite possible that the optimum amount of each was present in the original samples. However, as it would be impossible to remove these from saliva, no attempt was made to do so.

In view of the fact that the synthetic vitamin K completely inhibited the reaction, it would be interesting to know if this action has any relation to its vitamin K activity. For this reason, some of the extremely unstable natural vitamin K₁ was synthesized and tested. Due to the cost and difficulty of synthesis of this compound, the effect was tried in only 1 concentration—500 mg. per 100 cc. A definite but incomplete inhibition was observed. The 500 mg. per 100 cc. caused an inhibition of over 50%. This would indicate that the 2 actions are not closely related. Furthermore, we have since tested many other anti-oxidants, several of which were also tested by Armstrong (18). Many of these compounds act similarly to 2-methyl-1,4-naphthoquinone.

Cholesterol, which is now considered as having vitamin properties, usually showed a stimulation of acid production. It is interesting to note that Krasnow (19) found a definite trend toward high cholesterol concentrations in the saliva of caries active individuals.

SUMMARY

The effect of most of the known vitamins on acid formation from fermentable sugars in saliva has been studied. Only cholesterol, "Cerophyl", a mixture of vitamins, and possibly thiamin stimulated acid formation. Synthetic vitamin K (2-methyl-1,4-naphthoquinone) and natural vitamin K₁ (2-methyl-3-phytyl-1,4-naphthoquinone) inhibited acid formation. There was no indication that this action has any relationship to its vitamin K activity.

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THE SECOND TYPE OF BACTERIAL THIAMINASE*

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The second thiamine-decomposing bacterium, discovered by Kimura *et al.* (1, 2) was named by the Vitamin B Research Committee *Bacillus aneurinolyticus* Kimura *et* Aoyama (BKA) (3). By studying the properties of the enzyme produced by this bacillus, it was found that the action mechanism of the enzyme is completely different from that of *Bacillus thiaminolyticus* Matsumura *et* Misawa (BMM), shell-fish, fish and plant sources, which have been studied so far.

EXPERIMENTAL METHODS

For studying the effects of various amines on the enzyme, the properly diluted enzyme solution was incubated at 60° for one hour at pH 7 with 1 γ of thiamine in a total volume of 20 ml. The amines were used in a final concentration of 10^{-3} M. The remaining thiamine was estimated by the thiochrome method. The whole procedure was the same as was described in a previous paper (4).

For detecting the pyrimidine and thiazole derivatives, paper chromatography was used. In many cases the fluid, adjusted to pH 6.5, was dried on the paper strip and developed with acetic acid-n-butanol-water (1:1:5).

For detecting thiamine, heteropyrithiamine and quinothiamine, the fluorescence at the ultraviolet irradiation was used. For detecting other substances, Dragendorff reagent, as was used by Zaffaroni (6) was found to be useful.

In many base-substituted pyrimidine derivatives the spots tailed and the R_F values were not clear. In such cases the fluid to be tested was made up to 3 N in final concentration with HCl solution. After development the spots became very sharp and the R_F values could easily be determined. It is noteworthy that the R_F values of thiazole derivative and pyrimidinemethylaniline moved very markedly to the lower R_F side after acidification. Some R_F values of the pyrimidine and thiazole derivatives are shown in Table I.

* The results of this paper were presented before the Second International Congress of Biochemistry in Paris on July 22, 1952.

Table I

R_F Values of Pyrimidine and Thiazole Derivatives.

A: pH 6.5. B: 3 N HCl

Developing solution: Acetic acid-n-butanol-water (1:4:5).

Detecting solution: Dragendorff reagent or fluorescence.

Pm: 2-methyl-4-amino-pyrimidine

O, orange. r, red. v, violet. fl, fluorescence.

Substance	A		B	
	R _F	Color	R _F	Color
Thiamine	0.22-0.25	or	0.17-0.19	r
Pm H	0.43	vr	0.37	vr
Pm CH ₂ OH	0.32-0.38	vr	0.27-0.34	r
Pm CH ₂ NH ₂	0.16	vr		
Pm CH ₂ NH-CHO	0.38	vr		
Pm CH ₂ -Aniline	0.72-0.75	vr	0.43-0.46	vr
Pm CH ₂ - <i>o</i> -Nitroaniline	0.72	or	0.68	o
Pm CH ₂ - <i>m</i> -Nitroaniline	0.64	or	0.64	o
Pm CH ₂ - <i>p</i> -Nitroaniline	0.62	or		
Pm CH ₂ -Quinoline	0.28-0.33	fl		
Pm CH ₂ -Pyridine	0.23	fl		
2-Methyl-5- <i>o</i> -hydroxy-ethyl thiazole	0.92	rv	0.36-0.39	rv

EFFECT OF VARIOUS AMINES

As was previously reported (3), the enzymic activity in the supernatant of the ordinary broth culture at 37° rises remarkably after 3 days and reaches

Table II

*Effect of Various Amines.*pH 7, 60°, 1 hour. The final concentration of the amines was 10⁻³ M.

Pm, pyrimidine.

Substance	Per cent activation
Aniline	-30.2
Pyridine	0
Quinoline	-78.8
Glutamic acid	-3
<i>o</i> -Nitroaniline	-11.6
<i>m</i> -Nitroaniline	-14.5
<i>p</i> -Nitroaniline	-32.2
<i>o</i> -Aminobenzoic acid	0
<i>m</i> -Aminobenzoic acid	0
<i>p</i> -Aminobenzoic acid	0
<i>o</i> -Methoxyaniline	-63.8
2-Methyl-5- <i>o</i> -hydroxyethyl thiazole	-63.8
2-Methyl-4-amino-5-hydroxymethyl-Pm	-100
2-Methyl-4-amino-Pm	-87.2
2-Methyl-4-hydroxy-5-hydroxymethyl-Pm	-50.8
2-Methyl-4-hydroxy-Pm	-23.4
2-Aminopyridine	-11.2
2-Aminothiazole	-8.5
Hemosulfanilamide	0
Sulfanilamide	0
Cysteine	0
Glutathione	0

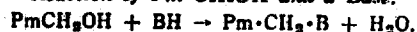
experiments with enzyme solution instead of washed bacterial cells showed equally the inhibitory effect of aniline on the enzyme. The test for the correlation of various molar ratios of aniline to thiamine to the formation of pyrimidinemethylaniline as well as the degree of activation by aniline showed, as can be seen from Table V, that pyrimidinemethanol was formed in low aniline concentration, while pyrimidinemethylaniline was formed in aniline concentration higher than twice that of thiamine.

FORMATION OF BASE-EXCHANGED PYRIMIDINE DERIVATIVE FROM PYRIMIDINEMETHANOL AND A BASE

The test for the formation of a base-exchanged derivative from pyrimidine-methanol and a base, resulted as shown in Table VI and demonstrated that

Table VI

Reaction of $\text{Pm}\cdot\text{CH}_2\text{OH}$ and a Base.



$\text{Pm}\cdot\text{CH}_2\text{OH}$, 5 mg. BH, 50 mg. 37° , 24 h. Th, 4-methyl-5- β -hydroxyethyl thiazole

BH	Formation of $\text{Pm}\cdot\text{CH}_2\cdot\text{B}$	
	BKA	BMM
Aniline	+	—
Pyridine	—	—
Quinoline	—	—
Th. *	—	—

* 25 mg of Th was used to 5 mg of $\text{Pm}\cdot\text{CH}_2\text{OH}$

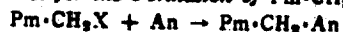
the base-exchanged derivative was formed only in the case of aniline. No other bases formed the pyrimidine derivatives. In the case of the BMM enzyme no formation of pyrimidine derivatives from any base and pyrimidinemethanol was demonstrated.

SUBSTRATES FOR THE FORMATION OF PYRIMIDINEMETHYLANILINE

Several derivatives of pyrimidinemethanol were tested as the substrate for the formation of pyrimidinemethylaniline in the presence of aniline. As shown in Table VII, pyrimidinemethanol can be replaced by pyrimidinemethylamine but not by the substituted pyrimidinemethylamine.

Table VII

Substrates for the Formation of $\text{Pm}\cdot\text{CH}_2\cdot\text{An}$



$\text{Pm}\cdot\text{CH}_2\text{X}$, 5 mg. An, 50 mg. pH 6.5, 37° , 40 h.

X	Formation $\text{Pm}\cdot\text{CH}_2\cdot\text{An}$	
	BKA	BMM
OH	+	—
NE	+	—
NE ⁺ CH ₃	—	—

DECOMPOSITION OF BASE-EXCHANGED PYRIMIDINE DERIVATIVES

It was observed that thiamine was formed as the reverse reaction of thiaminase by incubating the BMM enzyme with the base-exchanged pyrimidine derivative and the thiazole moiety of thiamine (4). Therefore, an experiment was made to test if the reverse reaction of the BKA enzyme is also possible by incubating the enzyme with pyrimidinemethylaniline and it was found that the reaction really takes place as shown in Table VIII, contrary to the case of

Table VIII

Decomposition of Base-Exchanged Pyrimidine Derivatives.



Pm-CH ₂ B	BH	Formation of Pm-CH ₂ OH	
		BKA	BMM
Anilinothiamine	Aniline	+	-
Heteropyrithiamine	Pyridine	+	-
Quinothiamine	Quinoline	+	-

the BMM enzyme. In the case of pyridine and quinoline, the reverse reaction of the above was not demonstrated (Table VI).

DISCUSSION

From the above findings it is evident that the thiaminase of BKA is entirely different from the known thiaminases of various origins. The chief differences are summarized in Table IX. The most remarkable difference is

Table IX

Comparison of the BKA Enzyme with the BMM Enzyme.

	BKA	BMM
Optimum temperature	60°	30°
Optimum pH	about 8	5
Condition of inactivation	60°, 20 min.	100°, 20 min.
Activation by bases	none or inhibitory	remarkable activation
Pm-CH ₂ OH formation by thiamine decomposition	+	-
Pm-CH ₂ -B formation from thiamine + base (except aniline)	-	+
(aniline)	+	+
Pm-CH ₂ -An formation from Pm-CH ₂ OH + Aniline	+	-
Pm-CH ₂ OH formation from Pm-CH ₂ -B	+	-

the failure of the base exchange reaction and that the decomposition products of thiamine are pyrimidinmethanol and the thiazole moiety, i. e. this enzyme is the real thiamine-hydrolyzing enzyme and the thiaminases, which have been studied in the past are in reality the base exchanging enzymes of thiamine. For convenience sake, we called the new type of thiaminase in BKA enzyme "Thiaminase II". In contrast to the known type of thiaminases which may now

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THE COURSE OF VITAMIN B₁ - RESORPTION

IN THE RAT.

German Academy of Sciences, Berlin; Institute for Nutrition,
Postdam-Rehbrücke; study range: physiology of nutrition.

With 5 figures in text.

(Received on November 25, 1959)

How far thiamine synthesized by the intestinal bacteria can be utilized depends on the resorption capacity of the intestine's distal section, besides its dependence on the bond with the bacterium cell and bacterial destruction in the intestine. The literature on thiamine resorption offers contradictory test results, all of which were obtained in capacity experiments with oral, rectal or intracecal application (1, 2, 7, 11, 13 - 15, 17, 19). We therefore attempted to clarify the course of the resorption and the resorption efficiency of single intestinal sectors with the aid of ³⁵S-thiamine.

Test methodology

A.1) 42 female rats, 8 weeks old, weight 100 g, of the Wistar type from our own breed received, 24 hours after withholding of the breeding diet, 1 ml of 15 μ C ³⁵S-thiaminebromidehydrobrominal in 0,01 n hydrochloric acid solution, with an activity of 15 μ C, administered with the gastric probe. After 10, 20, 30, 60, 120, 240 and 480 minutes, 6 animals each are killed under etherization and exsanguinated. The entire gastro-intestinal tract and the liver are prepared for examination; we thereby subdivided the small intestine into four equally long sections. After desiccation at 105 °, we incinerate the organs (aliquot parts of the liver) according to the LIDARN and LIGARD procedure, (12), modified by HENRICKS (5). We precipitate the radioactive sulfate as barium salt, whereby we add vehicles for the purpose of measurements in the vicinity of the infinite layer thickness zone. The measuring values obtained with a bell-type thin aperture (Geiger) counter are rectified regarding self-absorption, preparation distance and activity drop.

2) 4 groups of 3 animals each are kept in metabolism cages under the same application conditions; feces and urine are collected separately in the course of 24 hours. 8 hours after the thiamine administration the animals are given free access to the usual breeding fodder; the latter consists of 16,5% rye grist, 16,5 % barley grist, barley bread, 6,5 % wheat bran, 33,0 % boiled potatoes, 26,5 % boiled centrifuged pulp and 1 % sodium chloride. According to the operating procedure indicated above, the activity of the radioactive sulfur in the urine and feces is measured and the average per animal is computed.

3) Following oral administration of thiamine, we observed the ^{35}S blood level for 8 hours in 6 animals by currently taking 0,2 ml blood from the sinus retroorbitalis 1); the sulfur activity was subsequently determined with the specified method. For the purpose of translation into total blood (values), a blood volume of 7 ml serves as a basis for animals with a weight of 100 g, according to the data of BELCHER and HARRIS (3).

B. 24 animals, selected and prepared as under A 1), individually received during etherization 15 μg ^{35}S -thiaminebromidehydrobromide according to following laparotomy, injected into the cecum 1). After 20, 60, 240 and 480 minutes we killed 6 animals each; as after oral application, the gastro-intestinal tract and liver were removed and blood samples were taken; processing followed.

C. 42 rats of the same age, sex and weight as in Test A1) received 15 μg ^{35}S -thiaminebromidehydrobromide in 0,5 ml physiologic common salt solution, injected into the tail vein, without withholding fodder before or after the application. After 10, 20, 30, 60, 120, 240 and 480 minutes, they are killed in groups of 6; blood, stomach, intestine and liver were removed here as well. Before processing the organs with the customary method, we carefully removed part of the content from the single intestine sectors; the content is dried in the same way as the organ sectors, incinerated and examined for sulfur activity.

1) we are especially grateful for surgery performed by Dr. habil KERZ.

D. Working sequence example. Dry weight of the 4 small intestine sectors of 3 rats: 0,394 g. after incineration in the 50 ml KJELDAHL-flask (with short neck) on sand bath with 3 ml 70% HClO₄ and 1 ml BENE-IKT reagent (20 % Cu(NO₃)₂, 5 % Na ClO₃ in w.), the clear blue solution is evaporated and the residue is incorporated into 11 ml 3,5 % HCl-solution while heated. Vehicle additive: 0,25 ml nH₂S₄. The BaSO₄ is precipitated with 4 ml 5 % BaCl₂ solution and filtered out 12 hours later, using a synthetic suction filter. The filter with N. is stretched over a reversed polytyrol cup and dried under an infrared lamp. Weighed analytical product: 63,3 mg.

Activity measurement: probe diameter - 1,5 cm; diameter of the counter tube window (1,6 mg/cm²) = 3 cm; preparation distance = 3,3 cm; testing time: Jul 67, 1959, 10 a.m. Counted: 1800 imp. in 2 min = 900 imp/min.

Rectified: 1) Distance from preparation. At a distance of 3,3 cm, 19,75 % of the impulses determined at a distance of 1,3 were verified: $\frac{900 \cdot 100}{19,75} = 4556$ imp/min. 2) Self-absorption. With 63,3 mg BaSO₄, the self-absorption factor is 0,188 according to the calibration curve plotted under the same conditions. Accordingly, the result of the rectification is: $\frac{4556}{0,188} = 24230$ imp/min. 3) Activity drop since the start of the test on June 18, 7 a.m., time since start of experiment = 19,5 days, half-life period T = 87,1 days. $\frac{t}{T} = 0,2230 \frac{N}{N_0} = 15,6 \%$. $N_0 = \frac{24230 \cdot 100}{85,6} = 28510$ imp/min.

Specific activity relatively to the dry weight of the intestine is: 28510 : 0,394 = 72360 imp/min . g. The impulse count for the intestine section of a rat resulted from 28510 : 3, i.e. 9503 imp/min. Since under identical conditions 0,06 ug (0,06 µC) of the supplied thiamine results in 2995 imp/min and 15 µg, i.e. 743749 imp/min, an average of $\frac{9503 \cdot 100}{743749} = 1,27 \%$ of the applied dose is determined in each of the investigated intestine sectors. The values determined per rat are averaged with those of a parallel investigation in the course of

which the organs of 3 additional rats were processed according to the same method.

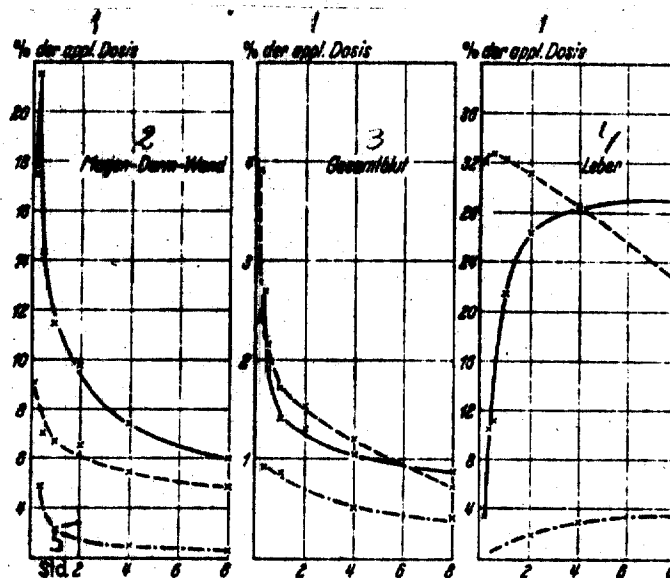
Results and Discussion

The chronological course of the activity in the gastro-intestinal wall, in the total blood volume and in the liver is shown in Figure 1 according to the percentage of the applied dose. With oral administration, maximal resorption is achieved after 20 minutes. Incorporation and demobilization occur parallelly in the liver thereby. As a result, the maximal content adjusts after 3 hours in asymptomatic approximation. On the other hand, the thiamine sulfur storage which follows the i.v. injection terminates already after 20 min.

After intracecal application, the maximal activity percentage in the gastro-intestinal wall is $\frac{1}{2}$, in the blood $\frac{1}{3}$ and in the liver $\frac{1}{7}$ of the maximal value in case of oral administration. The resorption capacity of the lower intestinal sectors is therefore substantially below the resorption capacity of the upper sectors.

In conjunction with this test, one of us (B) received an injection of 1 mg thiamine on the occasion of an appendectomy, administered into the cecum. Surplus excretion in the urine during the 24 hours following the injection indicates that merely 8 % of said dose was resorbed.

The summation of activity values found within 3 hours in the liver, intestine and blood results in 36 % of the orally applied radioactive thiamine sulfur. During that time, 15% appeared in the urine, and the non-resorbed quota excreted in the feces amounted to 3%. (Figure 2.)

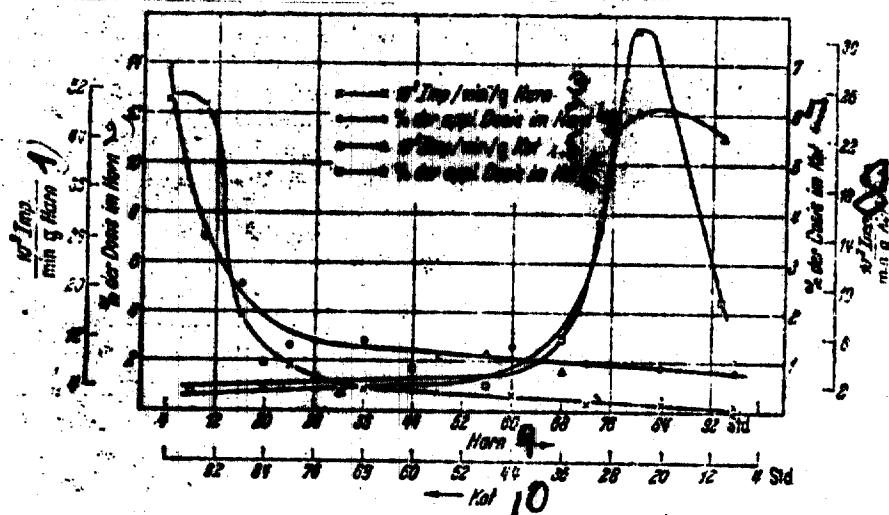


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FIGURE 1. S-thiamine utilization in the rat.

x—x oral, x-----x intavenous,
x- - - -x intracecal application)

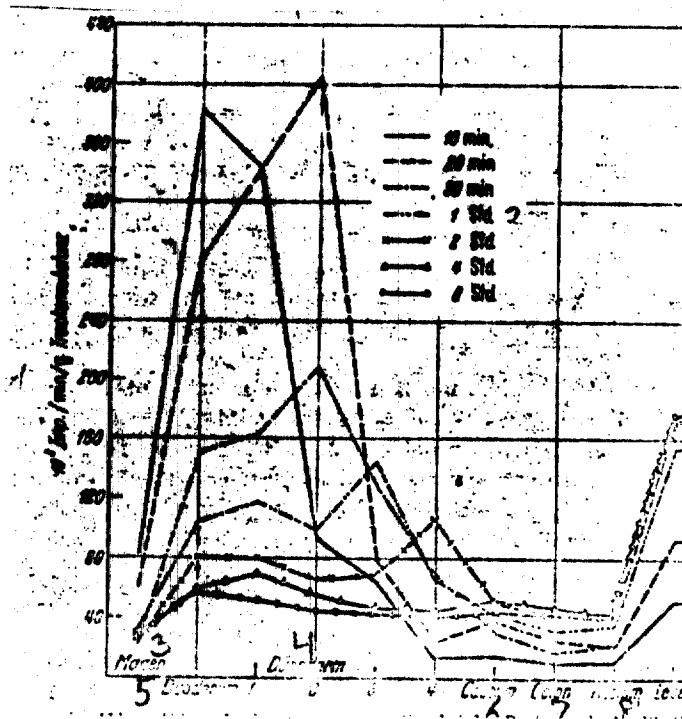
- 1) % of the applied dose; 2) gastro-intestinal wall;
3) total blood; 4) liver; 5) hours.



35

FIGURE 2 . Urine and feces excretion of S-activity with oral application of ^{35}S -thiamine .(Total excretion in 96 hours: 41,6 % in the urine and 14,5 in the feces).

- 1) $\frac{10^3 \text{ imp.}}{\text{min g urine}}$ 2) % of the dose in the urine;
3) $\frac{10^3 \text{ imp.}}{\text{min g}}$ in urine; 4) % of applied dose in the urine ;
5) $\frac{10^3 \text{ imp.}}{\text{min g}}$ in the feces ; 6) % of applied dose in the feces;
7) % of dose in the feces; 8) $\frac{10^3 \text{ imp.}}{\text{min g feces}}$; 9) urine; 10) feces.



35

FIGURE 3. Course of S-thiamine resorption in the rat; oral application.

- 3
- 1) 10 imp/min/g dry substance; 2) hours; 3) stomach;
 4) small intestine; 5) duodenum; 6) cecum; 7) colon;
 8) rectum; 9) liver.

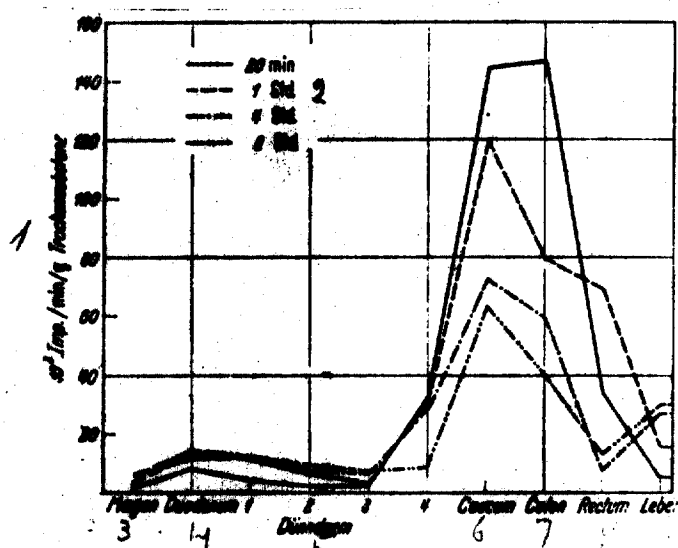
A total of 59 % of the administered volume is therefore determinable after 8 hours. The balance of 41% is presumably stored in the muscle system or is still in the intestines.

Figure 3 shows the activity measured at various points of time following oral thiamine ingestion, as a function of the weight unit of the dry stomach- and intestine wall as well as of the liver. In the individual wall sectors, said activity is obviously determined by local resorption in each case. The course of the curves and the chronological maximum deviation correspond to the intestinal transport.

The test reveals that the entire gastro-intestinal tract is capable of resorption, and that most of the thiamine is resorbed in the duodenum and in the small intestine. Activity values for the wall of the stomach, cecum, colon and rectum rose in the course of the experiment, but resorption efficiency is less significant under conditions which involve oral application. It is remarkable that a second activity maximum develops after a brief period of time in the duodenum and in the frontal small intestine.

When an identical thiamine dose is intravenously administered, the maximal activity value is verified in the duodenum wall 10 minutes after the injection; the test readings for said activity show bilateral decline which is steep relatively to the stomach, and gradual relatively to the colon and rectum. During the following period of time, all impulse counts regress, except for the stomach, whereby the activity is distributed in a comparatively regular pattern over the entire intestine; only the duodenum value stands out. The development of the activity which is balanced, except for the duodenum, proves that with enteral application, local resorption is the principal cause of activity concentrations in the wall of the digestive tract.

Impulse frequency likewise increases after intracecal administration whenever thiamine comes into direct contact with the intestinal wall after the occurrence of resorption : in the 4th small intestine sector, in the cecum, the colon and in the rectum (Figure 4).



35
 FIGURE 4. S-thiamine resorption in the rat, intracecal application.

3
 1) 30 imp/min/g dry substance ; 2) hours; 3) stomach; 4) duodenum; 5) small intestine; 6) cecum; 7) colon; 8) rectum; 9) liver.

The comparatively minor activity of the upper intestinal sections shows the same distribution as determinable in case of parenteral application. Maximal values in the cecum, the principal site of the resorption, are 2/3 lower than in the upper small intestine in case of oral ingestion. The test definitely indicates that the possible resorption efficiency of the single intestinal sections is determined by the available supply of resorbable thiamine.

The preferred involvement of the duodenum and of the frontal small intestine in activity distribution after intravenous or intracecal thiamine administration, as well as the second activity maximum which occurs there soon after oral application can be due either to intensified metabolic magnitude which parallels the resorption capacity, or they can be conditional on enterohepatic circulation.

an enterohepatic Vitamin B circulation was suspected earlier by MARIMURA (3). According to the investigations of BAGLIONI and GIARDINI (16), however, thiamine is not excreted with the bile. On the other hand, endogenous excretion in the intestine is believed to be certain. Thus MCCARTHY and collaborators (10) as well as WILBERT and CERECEDO (18) detected minor activity volumes in the feces of rats and rabbits respectively, after intramuscular and intravenous injection of ³⁵S-thiamine. Identical findings of JACONO and JOHNSON (6) and DRAVER (4) are available; they administered thiazole-¹⁴C-thiamine intraperitoneally to rats. We found during the examination of rat feces that the minor activity of the sulfur, as a function of the dry feces weight, remain near-constant 50 to 96 hours after oral application as well as 96 hours after intravenous application.

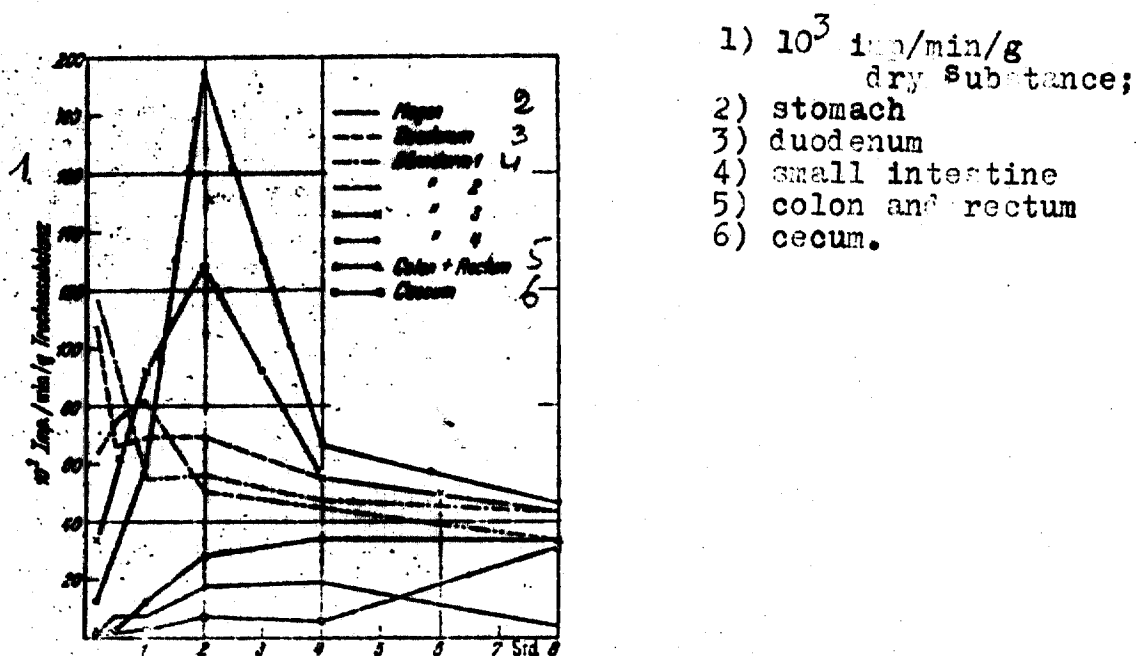


FIGURE 5. Radioactivity of the intestinal contents with i.v. injection of 15 μ Ci (15 μ Ci) ³⁵S-thiamine.

Figure 5 shows the readings of our test in the course of which the sulfur activity of the gastric and intestinal contents was observed after intravenous thiamine administration. The maximal activity occurs in the duodenum within the first ten minutes, and in the frontal small intestine within the first thirty minutes. During the following period of time the activity maximum progresses downwards in the small intestine, as it does after oral application. The above activity speaks in favor of the assumption that the thiamine sulfur passes through the small intestine and reaches the duodenum jointly with the bile through the ductus choledochus. Minor activity volumes are eliminated with the gastric juice as well; maximal values occur here between 2 and 4 hours. The impulse counts for the cecum contents show a pronounced elevation after 4 hours only, reaching the rectum after 8 hours. It remains questionable whether this phenomenon is ascribable to the delayed passage of the digested matter through the cecum, or whether the excretion of active substance in the colon and rectum is to be held responsible.

Findings concerning enterohepatic circulation are confirmed by a comparison of specific activities in the gastro-intestinal wall and the gastro-intestinal contents, following intravenous injection. While a concentration decline from contents to wall is present within the duodenum area and the area of the small intestine, the decline progresses from wall to contents in the stomach, cecum, colon and rectum (Table). Each gastro-intestinal section undoubtedly has its specific thiamine metabolism magnitude which possibly parallels the resorption capacity after oral thiamine ingestion.

we do not believe, however, that said metabolic magnitude is determinable after enteral application. We must assume instead on the basis of our test results, that activity distribution in the intestinal wall after parenteral administration occurs not only by means of the blood supply, but also forms through the superimposition of enterohepatic circulation.

TABLE. Radioactivity of the intestinal wall and intestinal contents,
average for a period of 8 hours after i.v. injection
of 15 μg (15 μC) ³⁵ S-thiamine.

Organ	³⁵ 10 imp/min/g dry substance	
	wall	contents
Stomach	17,6	9,5
duodenum	60,4	76,9
small intestine 1	50,6	68,9
small intestine 2	47,1	68,2
small intestine 3	45,5	70,2
small intestine 4	44,7	68,5
cecum	47,6	8,0
colon	41,2	
rectum	43,4	18,9

When evaluating present test results, it should be taken into consideration that only the radioactivity of the total sulfur volume was measured in all cases. The experiments are based on thorough studies, in an attempt to determine the specific radioactivity of thiamine as well as to identify various radioactive sulfur compound forms.

Biochemische Zeitschrift 332, 449—457 (1960)

Zum Ablauf der Vitamin-B₁-Resorption bei der Ratte

Von

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Mit 5 Textabbildungen

(Eingegangen am 25. November 1959)

Außer von der Bindung an die Bakterienzelle sowie der chemischen und bakteriellen Zerstörung im Darm hängt die Ausnutzbarkeit des von der Darmflora synthetisierten Thiamins von der Resorptionsfähigkeit der distalen Darmabschnitte ab. Die im Schrifttum über die Thiaminresorption vorliegenden Untersuchungsergebnisse, die alle in Belastungstests bei oraler, rektaler oder intracaecaler Applikation gewonnen worden sind, widersprechen einander [1, 2, 7—9, 11, 13—15, 17, 19]. Wir haben darum versucht, mit Hilfe von ³⁵S-Thiamin Aufschlüsse über den Ablauf der Resorption und die Resorptionsleistung einzelner Darmabschnitte zu gewinnen.

Versuchsmethodik

A. 1. 42 weiblichen, 8 Wochen alten und 100 g schweren Ratten vom Wistartyp eigener Zucht wird 24 Std nach Entzug des Zuchtfutters mittels Magensonde 1 ml einer 0,01 n salzsauren Lösung von 15 µg ³⁵S-Thiaminbromidhydrobromid mit einer Aktivität von 15 µC appliziert. Nach 10, 20, 30, 60, 120, 240 und 480 min werden jeweils 6 Tiere im Ätherrausch getötet und entblutet. Der gesamte Magen-Darm-Trakt und die Leber werden herauspräpariert; dabei unterteilen wir den Dünndarm in vier gleich lange Stücke. Nach dem Trocknen bei 105°C veraschen wir die Organe (von den Lebern aliquote Teile) nach dem von GESSIEKE [5] modifizierten Verfahren von ELDAAR u. NYGAARD [12]. Das radioaktive Sulfat fällen wir als Bariumsulfat, wobei wir Träger zusetzen, um nahe dem Bereich unendlicher Schichtdicke zu messen. Die mit einem dünnfenstrigen Glockenzähler erhaltenen Meßwerte werden hinsichtlich Selbstabsorption, Präparatabstand und Aktivitätsabfall korrigiert.

2. Unter denselben Bedingungen der Applikation werden 4 Gruppen von 3 Tieren in Stoffwechselkäfigen gehalten und im Verlauf von 96 Std Kot und Harn getrennt aufgefangen. Die Tiere erhalten 8 Std nach der Thiaminverabreichung freien Zugang zum gewohnten Zuchtfutter; dieses setzt sich aus 16,5% Roggenschrot, 16,5% Gerstenschrot, 6,5% Weizenkleie, 33,0% gek. Kartoffeln, 26,5% gek. Zentrifugenschlamm und 1% Natriumchlorid zusammen. Analog der oben angegebenen Arbeitsweise wird die Aktivität des Radioschwefels in Harn und Kot gemessen und das pro Tier bezogene Mittel berechnet.

3. Bei 6 Tieren verfolgen wir 8 Std den ³⁵S-Blutspiegel nach oraler Thiaminaufnahme, indem wir laufend 0,2 ml Blut aus dem Sinus retroorbitalis¹ entnehmen.

¹ Für die operativen Eingriffe sind wir Herrn Dr. habil. KETZ zu besonderem Dank verpflichtet.

und anschließend in der beschriebenen Weise die Schwefelaktivität ermitteln. Zur Umrechnung auf das Gesamtblut wird für die 100 g schweren Tiere nach den Angaben von BELCHER u. HARRISS [3] ein Blutvolumen von 7 ml zugrunde gelegt.

B. 24 Tieren gleicher Auswahl und Vorbereitung wie unter A1 werden im Ätherrausch nach Laparotomie 15 μ g 35 S-Thiaminbromidhydrobromid ins Caecum injiziert. Nach 20, 60, 240 und 480 min töten wir jeweils 6 Tiere, und wie nach der oralen Applikation werden Magen-Darm-Trakt und Leber sowie Blut entnommen und aufgearbeitet.

C. 42 Ratten gleichen Alters, Geschlechts und Gewichts wie im Versuch A1 injizieren wir jeweils 15 μ g 35 S-Thiaminbromidhydrobromid in 0,5 ml physiologischer Kochsalzlösung in die Schwanzvene, ohne ihnen vor- oder nachher Futter zu entziehen. Nach 10, 20, 30, 60, 120, 240 und 480 min werden stets 6 Tiere getötet, denen wiederum Blut, Magen, Darm und Leber entnommen werden. Ehe wir die Organe in gewohnter Weise aufarbeiten, präparieren wir aus den einzelnen Abschnitten des Verdauungstraktes vorsichtig einen Teil des Inhalts heraus; dieser wird wie die Organteile selbst getrocknet, verascht und auf Schwefelaktivität hin untersucht.

D. *Arbeitsbeispiel.* Trockengewicht der 4. Dünndarmwandabschnitte von 3 Ratten: 0,394 g. Nach der Veraschung im 50 ml-Kjeldahl-Kolben (mit kurzem Hals) auf dem Sandbad mit 3 ml 70%iger HClO_4 und 4 ml Benedikts Reagenz (20% Cu $[\text{NO}_3]_2$, 5% NaClO_3 in W.) werden die klare, blaue Lösung abgeraucht und der Rückstand unter Erwärmen in 11 ml 3,5%iger HCl aufgenommen. Trägerzusatz: 0,25 ml NH_4SO_4 . Das BaSO_4 wird mit 4 ml 5%iger BaCl_2 -Lösung gefällt und 12 Std danach unter Anwendung einer Kunststoffnutsche abfiltriert. Das Filter mit dem N. wird über ein umgekehrtes Polystyrolschälchen gespannt und unter einer Infrarotlampe getrocknet. Auswaage: 63,3 mg.

Aktivitätsmessung: Probendurchmesser = 1,5 cm, Durchmesser des Zählrohrenfensters ($1,6 \text{ mg/cm}^2$) = 3 cm. Präparatabstand = 3,3 cm. Meßzeit: 7. 7. 1959, 19.00 Uhr. Ausgezählt werden 1800 Imp. in 2 min = 900 Imp/min.

Korrekturen: 1. Präparatabstand. Bei 3,3 cm Abstand werden 19,75% der bei 1,3 cm Abstand gemessenen Impulse erfaßt: $\frac{900 \cdot 100}{19,75} = 4556 \text{ Imp/min}$. 2. Selbst-

absorption. Bei 63,3 mg BaSO_4 beträgt der Selbstabsorptionsfaktor nach einer unter denselben Meßbedingungen aufgestellten Eichkurve 0,188. Somit ergibt die

Korrektur $\frac{4556}{0,188} = 24230 \text{ Imp/min}$. 3. Aktivitätsabfall seit Versuchsbeginn am 18.6., 7.00 Uhr. Zeit seit Versuchsbeginn $t = 19,5$ Tage. Halbwertszeit $T = 87,1$ Tage.

$$\frac{t}{T} = 0,2239, \frac{N}{N_0} = 85,6\%, N_0 = \frac{24230 \cdot 100}{85,6} = 28510 \text{ Imp/min.}$$

Die auf das Darmstück entfallende spezifische Aktivität beträgt $28510 : 0,394 = 72360 \text{ Imp/min g}$. Die Impulszahl für den Darmabschnitt einer Ratte folgt aus $28510 : 3, \text{ d. s. } 9503 \text{ Imp/min}$. Da 0,06 μ g (0,06 μ C) des verstoffelten Thiamins unter denselben, werden in jenen der untersuchten Darmwandabschnitte im Mittel $\frac{9503 \cdot 100}{748749} = 1,27\%$ der appl. Dosis nachgewiesen. Die pro Ratte erhaltenen Werte werden mit denen einer Paralleluntersuchung, in der die Organe von weiteren 3 Ratten in gleicher Weise aufgearbeitet worden sind, gemittelt.

Ergebnisse und Diskussion

Der zeitliche Verlauf der Aktivität der Magen-Darm-Wand, des Gesamtblutes und der Leber in Prozent der applizierten Dosis ist in Abb. 1 dargestellt. Bei oraler Aufnahme wird das Maximum der Resorption nach 30 min erreicht. In der Leber finden dabei Inkorporation und Demobilisierung nebeneinander statt, so daß sich nach 8 Std der Höchstgehalt

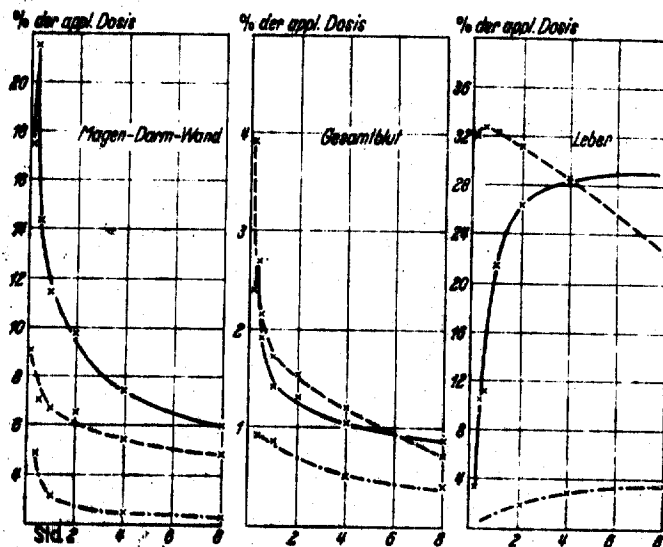


Abb. 1. Die ³²S-Thiaminschwefel-Resorption bei der Ratte. (x — x orale, x — x intravenöse, x — x intramuskuläre Applikation)

asymptotischer Näherung einstellt. Die der i.v. Injektion folgende Einlagerung des Thiaminschwefels in die Leber ist dagegen bereits nach 30 min abgeschlossen.

Nach intramuskulärer Applikation beträgt der höchste Prozentsatz der Aktivität in der Magen-Darm-Wand $\frac{1}{3}$, im Blut $\frac{1}{3}$ und in der Leber $\frac{1}{3}$ des Resorptionswertes der oralen Aufnahme. Die Resorptionsfähigkeit der unteren Darmabschnitte ist somit wesentlich geringer als die der oberen. Eine Bestätigung an diesem Versuch hat sich einer von uns (E.) anlässlich der Verabreichung von 1 mg Thiamin in das Cecum injizieren lassen. Aus der Aktivitätsbestimmung im Harn der folgenden 24 Std geht hervor, daß nur 8% des Thiaminschwefels resorbiert wurden.

Die Aktivitätswerte der in 8 Std gesammelten Aktivitätswerte von Leber, Blut und Harn betragen 30%, des oral verabfolgten Radiothiaminschwefels. In dieser Zeit 15% erschienen, und der mit dem Kot ausgeschiedene Anteil hat 8% betragen (Abb. 2).

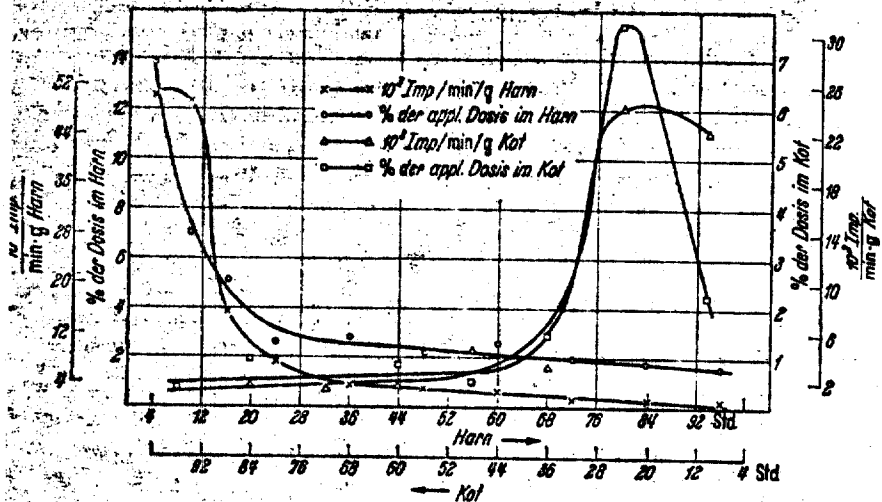


Abb. 2. Die Harn- und Kotausscheidung an ^{35}S -Aktivität bei oraler Applikation von ^{35}S -Thiamin (Gesamtausscheidung in 96 Std: 41,6% im Harn und 14,5% im Kot)

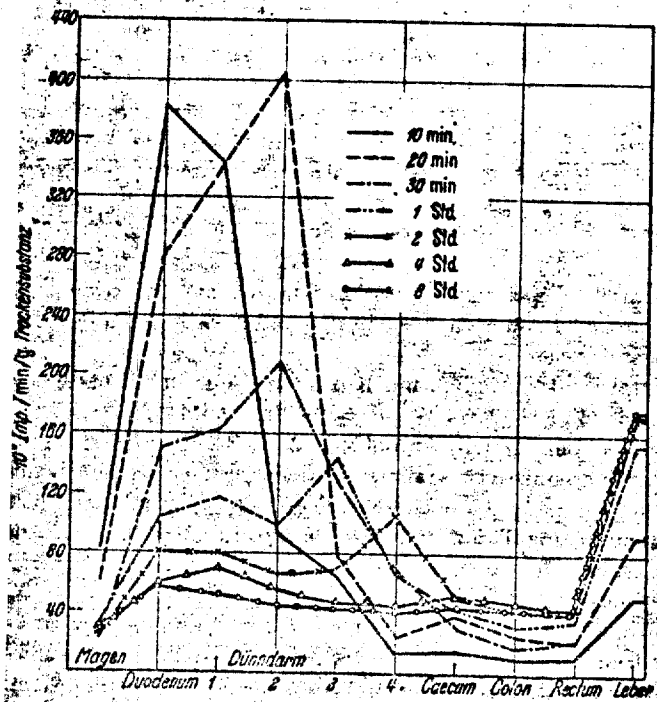


Abb. 3. Ablauf der ^{35}S -Thiaminresorption bei der Ratte, orale Applikation

Gesamt können somit nach 8 Std 59% der verabreichten Menge nachgewiesen werden. Die restlichen 41% dürften in die Muskulatur eingelagert werden oder sich noch im Darminhalt befinden.

In der Abb. 3 ist die zu verschiedenen Zeiten nach oraler Thiamin-aufnahme gemessene Aktivität, bezogen auf die Gewichtseinheit der trockenen Magen- und Darm-Wand sowie der Leber, dargestellt. Sie wird

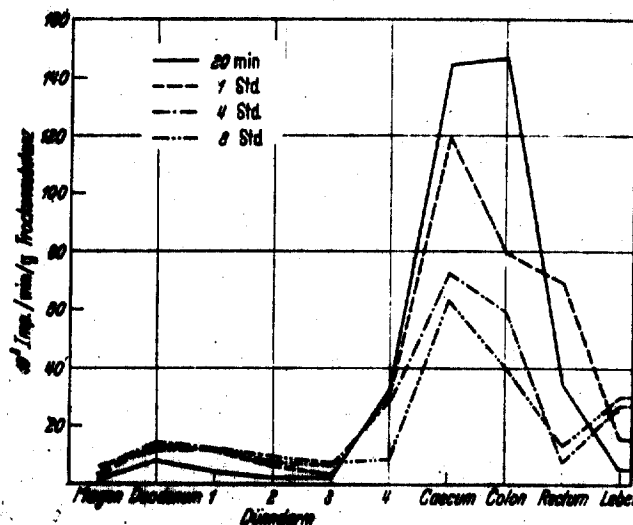


Abb. 4. Ablauf der ¹²⁵I-Thiaminresorption bei der Ratte, intracessale Applikation

in den einzelnen Wandabschnitten offensichtlich durch die jeweilige lokale Resorption bestimmt. Der Verlauf der Kurven und die zeitliche Maximalverschiebung entsprechen dem intestinalen Transport. Der Vergleich lehrt, daß der gesamte Magen-Darm-Trakt zur Resorption befähigt ist und daß die überwiegende Menge des Thiamins im Duodenum und im Dünndarm resorbiert wird. Die Aktivitätswerte von Magen-, Caecum-, Colon- und Rectumwand steigen zwar im Verlauf des Versuchs an, die Resorptionsleistung ist unter den Bedingungen der oralen Applikation aber von geringerer Bedeutung. Auffallend ist, daß sich schon nach kurzer Zeit für das Duodenum und den vorderen Dünndarm ein zweites Aktivitätszentrum herausbildet.

Bei intravenöser Thiamindosis intravenös verabfolgt, stellt man 10 min nach der Injektion in der Wand des Duodenum den höchsten Aktivitätswert fest. Abwärts davon fallen die Meßergebnisse — zum Magen steil ab. Vom Colon-Rectum hin abnehmend — ab. Im weiteren Zeitverlauf sinken die Aktivitätswerte im Magen genommen, alle Impulszahlen zurück, wobei sich

JACOBO u. JOHNSON [8] sowie von DRAPER [4] vor, die Ratten intraperitoneal Thiazol-¹⁴C-thiamin verabfolgt haben. Bei unseren Untersuchungen des Rattenkots haben wir festgestellt, daß die geringe Aktivität des Schwefels, bezogen auf das Kottrockengewicht, sowohl 24–96 Std nach oraler Applikation, als auch 96 Std lang nach intravenöser Injektion nahezu konstant bleibt.

In der Abb. 5 sind die Meßergebnisse unseres Versuchs dargestellt, bei dem nach der intravenösen Thiaminverabreichung die Schwefelaktivität des Magen- und Darminhalts verfolgt worden ist. Die höchste Aktivität liegt im Duodenum innerhalb der ersten zehn und im vorderen Dünndarm innerhalb der ersten 30 min vor. Im weiteren Zeitverlauf wandert das Aktivitätsmaximum ebenso wie nach oraler Aufnahme den Dünndarm abwärts. Dieser Aktivitätsverlauf spricht für die Dünndarmpassage von Thiaminschwefel, der mit der Galle über den Ductus choledochus in das Duodenum gelangt. Geringe Aktivitätsmengen werden auch mit dem Magensaft abgesondert; Höchstwerte treten hier zwischen 2 und 4 Std auf. Die Impulszahlen des Blinddarminhalts steigen erst nach 4 Std deutlich an, um nach 6 Std die des Enddarmes zu erreichen. Es bleibt fraglich, ob diese Erscheinung auf eine verzögerte Passage des Thiamins durch das Caecum oder eine Exkretion von aktiver Substanz in Colon und Rectum zurückzuführen ist.

Tabelle. Radioaktivität von Darm-Wand und -Inhalt im Mittel über eine Zeit von 8 Std nach i. v. Injektion von 15 µg (¹⁴C) ¹²⁵I-Thiamin

Organ	10 ³ Imp./min/g Trockensubstanz	
	Wand	Inhalt
Magen	17,6	9,5
Duodenum	60,4	76,9
Dünndarm 1	50,6	68,9
Dünndarm 2	47,1	69,2
Dünndarm 3	45,5	70,2
Dünndarm 4	44,7	68,5
Caecum	47,6	8,0
Colon	41,2	18,9
Rectum	43,4	

Die erhobenen Befunde über einen enterohepatischen Kreislauf werden bestätigt durch eine Gegenüberstellung der spezifischen Aktivitäten von Magen-Darm-Wand und Magen-Darm-Inhalt nach intravenöser Injektion. Im Bereich des Duodenums und des Dünndarms ein Aktivitätsgefälle vom Inhalt zur Wand besteht, liegt für Magen, Colon und Rectum ein Gefälle von der Wand zum Inhalt vor.

Sicher hat jeder Magen-Darm-Abschnitt seine besondere Thiaminabsorption, die möglicherweise der Resorptionsleistung nach der Nahrungsaufnahme parallel geht. Wir glauben jedoch nicht, daß die Absorption nach enteralem Angebot erkennbar ist. Wir können daher auf Grund unserer Versuchsergebnisse annehmen, daß Thiamin nach parenteraler Aufnahme

...auf dem Wege der Blutversorgung sondern auch durch Über-
...eines entzündlichen Kreislaufes zustande kommt.

...der Beurteilung der vorgelegten Untersuchungsergebnisse muß
...werden, daß in allen Fällen nur die Radioaktivität des
...gewonnen worden ist. Die Versuche sind der Ausgang
...Bearbeitungen, bei denen neben der Ermittlung der spezi-
...Radioaktivität des Thiamins der Organe eine weitgehende Tren-
...und Identifizierung der verschiedenen Verbindungsformen des
...Schwefels versucht werden soll.

Zusammenfassung

Durch orale, intracaseale und intravenöse Applikation von ^{35}S -Thia-
min und Bestimmung der Radioaktivität des Schwefels in der Wandung
und im Inhalt des Verdauungstrakts, sowie in Blut, Leber, Harn und
Milch wird nachgewiesen, daß der gesamte Magen-Darm-Trakt zur Thiamin-
resorption befähigt ist. Nach entzialem Angebot reichert sich Thiamin
primär in der Wand des resorbierenden Darmteils an, wobei die Konzen-
tration lokal größer ist als nach parenteraler Verabfolgung. Bei oraler
Thiaminaufnahme erfolgt die Resorption im wesentlichen im Duodenum
und im Blinddarm. Die Resorptionsleistung der einzelnen Darmabschnitte
wird jedoch durch das Angebot an resorbierbarem Thiamin bestimmt.
Sie beträgt bei gleichem Angebot in den distalen Abschnitten nur etwa
20% derjenigen von Duodenum und Dünndarm. Die den jeweiligen
Intestinalabschnitten eigene Thiaminstoffwechselgröße ist nicht nach
...Angebot erkennbar, weil sich in einem enterohepatischen
...Blutversorgung eine zusätzliche Resorption überlagert.

Summary

^{35}S -thiamine was given to rats orally, intracaseally, and intravenously.
By determinations of the radioactivity in the intestinal wall and contents
as well as in the blood, liver, urine, and faeces it is demonstrated that
the entire intestinal tract is able of thiamine resorption. After the
oral administration thiamine is enriched primarily in the wall of the
resorbing intestinal tract. The local concentration was found to be
higher than after parenteral application. After oral thiamine
administration, resorption occurs mainly in the duodenum and small
intestine. The resorption capacity of the individual intestinal
segments is determined by the amount of resorbable thiamine. Under equal
conditions the distal segments resorb only about 20 per cent of
thiamine as compared to the proximal small intestine. The thiamine
exchange capacity of the individual intestinal segments cannot be determined after
...to the

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ALTERATION PRODUCTS OF THIAMINE IN INJECTABLE SOLUTIONS AND THEIR ACUTE AND SUB-CHRONIC TOXICITY

The easy alterability of certain drugs in solution, especially at the usual temperatures of the sterilization process poses not only the problem of maintaining the drug at the established level of concentration and thereby therapeutically useful (which sometimes is obtainable with a suitable overdosing), but also the one, often neglected, of alteration products, that, besides being generally unprepared of therapeutic action, can have a different toxicity, than the unaltered drug.

From this point of view we intend to study vitamins: for this purpose we, first of all, investigate thiamine or vitamin B₁.

Thiamine is sufficiently thermostable in the slightly acid solution (pH = 4 approx.); this stability decreases in a neutral environment and especially in alkaline, and also in a strongly acid environment.

To stabilize conditions not dissimilar to of injectable solutions, we have autoclaved a thiamine solution at pH 6.5. The characteristic products obtained by chromatography on the paper, we have tested, in their complex, the acute toxicity and that one sub-chronic in comparison with the unaltered thiamine.

Preparation of solutions. - The chlorinated thiamine F.U. of the Firm C. Erba was used. Three solutions of 10, 25 and 50mg/ml concentration were prepared which were brought to pH 6.5. Later solutions were put in phials (without azote). Certain phials were sterilized for tyndallization (5 times at intervals of 24 hours, one hour at 60°C; others were autoclaved for certain days (1 hour every day at 115°C) up to the titer in thiamine, determined by the spectrophotometric method at the

for urea and for glucose using reactive agents of the Boehringer Firm; the hemoglobinemia was determined colorimetrically after denaturation of the alkali.

The results are shown on Table 1. No cases showed significant statistically different results.

Conclusions. - The most interesting result of our experiment the minor toxicity of alteration products of the thiamine with respect to the unaltered vitamins. This is probably due to the fact, that in the latter in the thiazolic ring, quaternary ammonia function is present which, instead of becoming absent in 4-methyl-5-oxyethyl-thiazol forms for destruction of the thiamine.

One would therefore deduce that the thiamine toxicity would be mostly of the curaric type, i. e., connected to the quaternary azote; the thiaminic shock, instead, would be connected to the presence of pyrimidinic ring. However it can be excluded, that fatal cases sometimes verified in a man due to parenteral administration of elevated doses of vitamin B₁ could be attributed to vitamin alteration.

As far as sub-chronic toxicity is concerned, our opinion is that disagreement in the literature is due to the diversity of used techniques, to the insufficient number of treated animals; from our experiments it results that neither thiamine nor its alteration products modify in satisfactory manner the parameters taken in the investigation of rats.

TABLE 1.

Average values (\pm standard deviation) of certain parameters in treated rats with thermically altered thiamine or unaltered.

Measured parameters	Control rats	Rats injected with *	
		altered thiamine	unaltered thiamine
Initial weight (gr)	79.9 \pm 11.8	85.3 \pm 7.7	82.8 \pm 9.0
Ponderal increase after 42 days (gr).....	127.9 \pm 35.0	121.6 \pm 13.4	123.1 \pm 19.5
Weight % liver.....	2.87 \pm 0.14	2.87 \pm 13.4	2.76 \pm 0.13
" " spleen.....	0.15 \pm 0.08	0.15 \pm 0.06	0.15 \pm 0.06
" " kidneys.....	0.71 \pm 0.07	0.76 \pm 0.08	0.69 \pm 0.05
" " adrenal glands	0.021 \pm 0.003	0.020 \pm 0.002	0.021 \pm 0.004
" " testicles....	1.38 \pm 0.24	1.32 \pm 0.16	1.33 \pm 0.25
" " heart.....	0.27 \pm 0.03	0.29 \pm 0.05	0.28 \pm 0.03
" " thymus.....	0.18 \pm 0.06	0.19 \pm 0.06	0.18 \pm 0.05
Asotemia (N ureic) mg/100 ml....	16.3 \pm 1.8	15.0 \pm 3.1	15.7 \pm 2.8
Glycemia gr/1000 ml....	0.64 \pm 0.07	0.65 \pm 0.06	0.66 \pm 0.08
Hemoglobinemia gr/100 ml	16.7 \pm 1.6	17.4 \pm 2.0	16.9 \pm 1.2
Hematocrit.....	48.5 \pm 2.7	49.5 \pm 2.8	48.8 \pm 2.3
Erythrocytes (millions/mm ³)	7.86 \pm 0.75	7.99 \pm 0.66	7.87 \pm 0.41
Leucocytes (per mm ³)	9107 \pm 945	7933 \pm 1760	9121 \pm 920

* Rats in groups of 15, were injected daily, with 42 gr., via intramuscular, with 0.2 ml/ (5 mg) of thiamine altered or unaltered.

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Prodotti di alterazione della tiamina nelle soluzioni iniettabili e loro tossicità acuta e sub-cronica

La facile alterabilità di alcuni farmaci in soluzione, specialmente alle temperature comunemente usate nei processi di sterilizzazione, pone non solo il problema di mantenere il farmaco al livello dichiarato di concentrazione e quindi terapeuticamente utile (il che è talvolta ottenibile con un opportuno sovradosaggio), ma anche quello, spesso trascurato, dei prodotti di alterazione, che, oltre ad essere generalmente sprovvisti di azione terapeutica, possono avere una loro tossicità, diversa da quella del farmaco inalterato.

È nostro intendimento studiare da questo punto di vista le vitamine: a tale scopo abbiamo per prima cosa preso in esame la tiamina o Vitamina B₁.

La tiamina è abbastanza termolabile in soluzione leggermente acida (pH intorno a 4); la stabilità diminuisce in ambiente neutro e soprattutto alcalino, ed anche in ambiente fortemente acido.

Per stabilire condizioni non dissimili da quelle che possono aver luogo nelle soluzioni iniettabili, abbiamo autoclavato una soluzione di tiamina a pH 6,5. Caratterizzati i prodotti ottenuti mediante cromatografia su carta, ne abbiamo saggiato, nel loro complesso, la tossicità acuta e quella subcronica in confronto con quella della tiamina inalterata.

Preparazione delle soluzioni. — Si è usata tiamina cloridrato F.U. della Ditta C. Erba. Se ne sono preparate tre soluzioni alle concentrazioni di 10, 25 e 50 mg/ml, che si sono portate a pH 6,5. Le soluzioni sono state poi infilate (senza aereo). Alcune fiale sono state sterilizzate per tindalizzazione (5 volte, a distanza di 24 ore, per 1 ora a 60°C; altre sono state autoclavate per vari giorni (ogni giorno per 1 ora a 115°C) finché il titolo in tiamina, determinato col metodo spettrofotometrico al tiocromo¹, risultava di non oltre il 2 % di quello iniziale. Nelle fiale tindalizzate il titolo restava pressoché inalterato.

Esame cromatografico. — Le soluzioni alterate, notevolmente colorate in giallo, sono state sottoposte a cromatografia su carta usando carta Whatman n. 1 e, come fase mobile, la miscela n-butano-etanolo-acqua nel rapporto 2:1:1² o 2:2:1; quest'ultima dà una risoluzione migliore. L'identificazione dei componenti è stata fatta esaminando i cromatogrammi a luce U.V. di bassa lunghezza d'onda (lampada germicida della General Electric Co. schermata con filtro Corning n. 9863), spruzzandoli poi con i reattivi usati da LHOEST, BUSSE & BAUMANN³ e da WINDHEUSER & HIGUCHI⁴, ed eseguendo cromatogrammi con sostanze di confronto.

Il secondo solvente ha permesso di evidenziare, nelle soluzioni alterate, almeno 5 prodotti: 2-metil-4-ammino-5-amminometil-pirimidina (Rf 0,42), tiamina (Rf 0,62), 2-metil-4-ammino-5-ossimetil-pirimidina (Rf 0,77), tiocromo (Rf 0,82), 4-metil-5-ossimetil-tiazolo (Rf 0,91); si vedono anche piccole quantità di altri prodotti, alcuni dei quali fluorescenti, non ben differenziati, con Rf inferiore a 0,4.

Tossicità acuta. — È stata saggiata in topini bianchi del peso di ca. 20 g. mediante inoculazione nella vena caudale. Dopo alcune prove orientative si sono iniettate a gruppi di 10 animali per ogni dosaggio quantità di tiamina alterata fra 100 e 500 mg per Kg di peso corporeo; la DL₅₀ è risultata pari a 260 mg/kg (Fig. 1). Prove parallele

eseguite con tiamina integra hanno dato una DL_{50} di 125 mg/kg. in perfetto accordo con la letteratura ⁴.

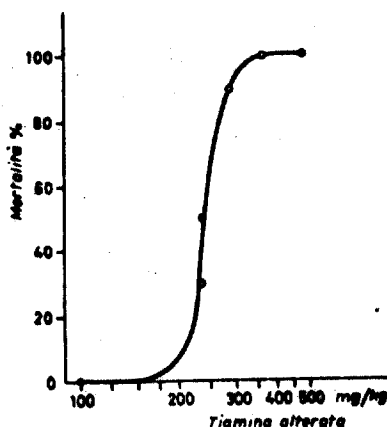


Fig. 1. — Curva dose/mortalità nel topo per i prodotti di demolizione termica della tiamina. (Autoclavata a 115°, ogni giorno per un'ora, fino a titolo fotometrico 1 del 5%).

Tossicità subcronica. — È stata saggiata in ratti albini (ceppo Wistar) di sesso maschile del peso iniziale intorno a 80 g. Ad un lotto di 15 animali sono stati iniettati giornalmente, per 42 giorni consecutivi, per via intramuscolare, 0,2 ml (= 5 mg) di soluzione di tiamina alterata; ad un altro lotto di 15 animali è stata iniettata la stessa quantità di tiamina inalterata; ad un terzo egual lotto (controlli) si è iniettata una soluzione fisiologica. La dieta era per tutti quella standard per ratti usata nel nostro Istituto.

Durante l'esperimento i ratti non hanno mostrato alcun fenomeno anormale. Alla fine gli animali, tenuti 12 ore a digiuno, sono stati pesati, inoculati i. p. con 0,2 ml di soluzione di eparina all'1 %, e, dopo circa 1 ora, sacrificati. Sono stati pesati fegato, milza, reni, surreni, testicoli, cuore, timo; si sono inoltre determinati azotemia, glicemia, emoglobinemica, ematocrito, eritrociti e leucociti. Azotemia e glicemia sono state determinate con i metodi enzimatici specifici per l'urea e il glucosio, usando i reattivi della ditta Boehringer; l'emoglobinemica è stata determinata colorimetricamente dopo denaturazione alcalina.

I risultati sono raccolti nella Tabella 1. In nessun caso le differenze sono risultate statisticamente significative.

Conclusioni. — Il risultato più interessante dei nostri esperimenti è la minore tossicità dei prodotti di alterazione della tiamina rispetto a quella della vitamina inalterata. Ciò è dovuto probabilmente al fatto che in quest'ultima è presente, nell'anello tiazolico, la funzione ammonica quaternaria, che è invece assente nel 4-metil-5-ossietil-tiazolo formato per demolizione della tiamina. È interessante a questo proposito rilevare che la ditiopropiltiamina, nella quale l'anello tiazolico è aperto, ha una tossicità da 2 a 5 volte minore di quella della tiamina ^{4,5}.

Se ne potrebbe quindi dedurre che la tossicità della tiamina sia in gran parte di tipo curarico, cioè legata all'azoto quaternario; lo shock tiaminico, invece, sarebbe legato alla presenza dell'anello pirimidinico ⁷. Sembra pertanto di poter escludere che i casi mortali talora verificatisi nell'uomo in seguito a somministrazione parenterale di dosi elevate di Vitamina B₁ si possano imputare ai prodotti di alterazione della vitamina.

TABELLA 1.

Valori medi (\pm deviazione standard) di alcuni parametri
in ratti trattati con tiamina alterata termicamente o inalterata

Parametri misurati	Ratti di controllo	Ratti iniettati con *	
		tiamina alterata	tiamina inalterata
Peso iniziale (g)	79,9 \pm 11,8	85,3 \pm 7,7	82,8 \pm 9,0
Accrescimento ponderale dopo 42 giorni (g)	127,9 \pm 35,0	121,6 \pm 13,4	123,1 \pm 19,5
Peso % del fegato	2,87 \pm 0,14	2,87 \pm 0,27	2,76 \pm 0,13
• • della milza	0,15 \pm 0,06	0,15 \pm 0,06	0,15 \pm 0,06
• • dei reni	0,71 \pm 0,07	0,76 \pm 0,08	0,69 \pm 0,05
• • dei surreni	0,021 \pm 0,003	0,020 \pm 0,002	0,021 \pm 0,004
• • dei testicoli	1,38 \pm 0,24	1,32 \pm 0,16	1,33 \pm 0,25
• • del cuore	0,27 \pm 0,03	0,29 \pm 0,05	0,28 \pm 0,03
• • del timo	0,18 \pm 0,06	0,19 \pm 0,06	0,18 \pm 0,05
Azotemia (N ureico) mg/100 ml	16,3 \pm 1,8	15,0 \pm 3,1	15,7 \pm 2,8
Glicemia g/1000 ml	0,64 \pm 0,07	0,65 \pm 0,06	0,66 \pm 0,08
Emoglobinemica g/100 ml	16,7 \pm 1,6	17,4 \pm 2,0	16,9 \pm 1,2
Ematocrito	48,5 \pm 2,7	49,5 \pm 2,8	48,8 \pm 2,3
Eritrociti (milioni/mm ³)	7,86 \pm 0,75	7,99 \pm 0,66	7,87 \pm 0,41
Leucociti (per mm ³)	9107 \pm 945	7933 \pm 1760	9121 \pm 920

* I ratti, in lotte di 15, sono stati iniettati giornalmente, per 42 gg., per via intramuscolare, con 0,3 ml (5 mg) di tiamina alterata o inalterata.

Per quanto riguarda la tossicità subcronica, è nostra opinione che le discordanze della letteratura * siano dovute, più che alle diverse tecniche usate, all'insufficiente numero di animali trattati; dalle nostre esperienze risulta che né la tiamina né i suoi prodotti di alterazione modificano in maniera statisticamente significativa i parametri da noi presi in esame nel ratto.

9 maggio 1966.

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THE EFFECT OF THIAMINE CONSUMPTION ON LIVER MICROSOMAL DRUG-METABOLIZING PATHWAYS¹

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ABSTRACT

GROSSE, WILLIAM, III AND A. E. WADE: The effect of thiamine consumption on liver microsomal drug metabolizing pathways. *J. Pharmacol. Exp. Ther.* 176: 758-765, 1971. Male and female Sprague-Dawley rats were maintained on synthetic diets for three weeks. One diet was deficient in thiamine and the other was a high thiamine (HT) diet that supplied an average daily intake of 2.0 mg of thiamine to each animal. Rats receiving the HT diet showed significant reductions in aniline, soxasolamine and aminopyrine metabolic rates *in vitro*, whereas hexobarbital metabolic rate was not significantly altered. A reduction in hepatic microsomal cytochrome b₅ and cytochrome P-450 contents without a reduction in microsomal protein content was evident in rats fed the HT diet. As further evidence for this effect being in the microsomal fraction, increasing reduced nicotinamide adenine dinucleotide phosphate levels in the incubation mixtures failed to increase metabolic rates; furthermore, the 355,000 × g liver supernatant from rats fed the thiamine-deficient diet did not enhance the metabolic rate of microsomes from rats fed the HT diet; nor did the supernatant from animals fed the HT diet depress the metabolic rate of microsomes from the deficient group. Reduced nicotinamide adenine dinucleotide phosphate-cytochrome c reductase activity of microsomes from rats fed the HT diet was depressed.

Many factors have been shown to alter the metabolism of drugs by microsomal enzyme systems in rat liver. Apart from environmental states, hormones and other drugs, the diet and nutritional status of the animal can also markedly influence its capability to metabolize drugs. Kato (1967) showed that starvation of female rats increased their ability to metabolize hexobarbital, aminopyrine and aniline, whereas heavy sucrose feeding decreased metabolism of these substrates. Protein and lipid deficiency may also depress the rate of drug metabolism (Brown *et al.*, 1964; Kato *et al.*, 1962; Century and Horwitt, 1968; Weatherholla *et al.*, 1968; Marshall and McLean, 1969; Caster *et al.*, 1968, 1970; Wade *et al.*, 1969a). Certain vitamins, (Kalyanpur *et al.*, 1968; Harper and Calcutt, 1961; Wade *et al.*, 1969b) and vitamin deficiency states

(Conney *et al.*, 1961; Kato *et al.*, 1969) have also been shown to alter drug metabolism or duration of drug action.

The present investigation was undertaken to examine the effects of dietary thiamine upon drug-metabolizing pathways and to investigate various parameters essential to these pathways in an attempt to elucidate the mechanism by which thiamine alters metabolic rate.

METHODS. Male and female Sprague-Dawley rats (The Holtzman Co., Madison, Wisc.) weighing 50 to 60 g (21-23 days old) were stabilized for 4 to 7 days on Purina lab chow before being placed on the laboratory test diet for three weeks. The diet contained 2.2% thiamine-free vitamin fortification mix (Nutritional Biochemicals Corporation, Cleveland, Ohio), 3% corn oil, 4% Jones Foster salt mixture, 4% non-nutritive bulk (Alphacel, Nutritional Biochemicals Corporation), 16% vitamin-free casein and 70.8% sucrose. One diet was thiamine deficient (TD); the other was a high thiamine (HT) diet that supplied an average daily intake of 2.0 mg of thiamine HCl to each animal. All animals of like sex were caged in groups of two in an air-conditioned room.

Preparation of tissue samples. The rats were decapitated without anesthesia, livers were quickly removed and homogenates were made as described.

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by Wade *et al.* (1966b). Each homogenate was centrifuged at $9,000 \times g$ for 20 minutes at $0-4^{\circ}\text{C}$ in a Sorvall model RC-2B centrifuge, after which the supernatant containing the microsomes was decanted. In some experiments the $9,000 \times g$ supernatant was centrifuged at $105,000 \times g$ for one hour at $0-4^{\circ}\text{C}$ in a Beckman model L2-65 ultracentrifuge to obtain the microsomes. Microsomes were either resuspended in 1.15% KCl or in the $105,000 \times g$ supernatant from the liver of a rat receiving the alternate diet.

Enzyme assays. A typical incubation mixture contained the equivalent of 330 mg of liver, 1.0 μmol of nicotinamide adenine dinucleotide phosphate (NADP), 25.0 μmol of glucose-6-phosphate, 50 or 100.0 μmol of nicotinamide, 25.0 μmol of magnesium sulfate and sufficient 0.1 M phosphate buffer (pH 7.4) to make 5.0 ml. As drug substrate, the typical incubation mixture contained one of the following: 10.0 μmol of aniline, 3.0 μmol of soxazolamine, 2.0 μmol of hexobarbital or 40.0 μmol of aminopyrine. Incubation mixtures utilizing the $105,000 \times g$ microsomes also contained 2 enzyme units of glucose-6-phosphate dehydrogenase.

The mixture was incubated for 20 or 30 minutes at 37°C under air in a Dubnoff metabolic shaker, after which the reaction was stopped by chilling the incubation flasks in an ice bath. An appropriate aliquot of the incubate was then transferred to a tube containing the extraction medium.

The aromatic hydroxylation of aniline was determined by measuring *p*-aminophenol formed, utilizing a modification of the method reported by Kato and Gillette (1966). The aromatic hydroxylation of soxazolamine was determined by measuring the disappearance of the substrate according to the method of Conney *et al.* (1966). The aliphatic hydroxylation of hexobarbital was determined by measuring the disappearance of substrate according to the method of Cooper and Brodie (1955). The demethylation of aminopyrine was determined by measuring 4-aminoantipyrine formed, according to the method of La Du *et al.* (1955).

Reduced nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome *c* reductase activity of rat liver microsomes was determined by the method of Phillips and Langdon (1962). Glucose-6-phosphate dehydrogenase activities were determined for the $105,000 \times g$ supernatant from rat liver homogenates by the method of Glock and McLean (1963), with the Perkin-Elmer model 52 split-beam recording spectrophotometer at 40 m μ .

Aliesterase and acetylcholinesterase activities were determined titrimetrically by measuring the acetic acid liberated from the substrate α -naphthol acetate by aliesterase, or from acetylcholine perchlorate by acetylcholinesterase, with the Radiometer

automatic pH titrator. Reaction rates were calculated from initial rates recorded automatically.

Estimation of cytochromes P-450 and *b*. The cytochrome *b*₅ content of hepatic microsomes resuspended in 1.15% KCl containing 0.05 M Tris buffer (pH 7.4) at a constant protein concentration of 2.5 mg/ml was determined by the method of Omura and Sato (1964). The differences in the absorption spectra were measured at room temperature with the Amico-Chance dual wavelength/split beam recording spectrophotometer using the split beam mode, and were recorded on 10-inch rectilinear paper at a sensitivity of 0.5 (i.e., 10-inch pen deflection = 0.5 absorbance units). Cytochrome *b*₅ content was calculated with the extinction coefficient of 185 cm² mM⁻¹. The cytochrome P-450 content was calculated by the method of Omura and Sato (1964) with the extinction coefficient for cytochrome P-450 of 91 cm² mM⁻¹.

Sleeping and paralysis times. Hexobarbital sleeping time was measured from the time of i.p. injection of 125 mg/kg of hexobarbital sodium to recovery of the righting reflex. Duration of soxazolamine paralysis was determined from the time of i.p. injection of 100 mg/kg of soxazolamine to recovery of the righting reflex. To prevent malingering, the rats were touched on their tails once each five minutes.

Statistics. Calculation of V_{max} and K_m of aniline hydroxylase and hexobarbital oxidase was accomplished with the computer program of Wilkinson (1961). All data were analyzed by Student's *t* test, and $P < .05$ was taken as the minimal level of significance.

RESULTS. Body weights. Body weights of male and female rats fed the TD diet were significantly less than their controls after 14 days. Male rats fed the HT diet gained an average of 81 g, whereas those fed the TD diet gained only 8 g during the 21-day feeding period. During this time, the females fed the HT diet had gained an average of 65 g, whereas those fed the TD diet gained only 15 g.

Liver weights. Liver weights and their percentage of body weight of rats fed the HT and TD diets for 21 days are shown in table 1. Liver weight as a percentage of body weight was significantly higher in male rats fed the HT diet than in those fed the deficient diet, whereas that of female rats was not significantly different.

In vitro drug metabolism. Table 2 shows the rates of aniline, soxazolamine, hexobarbital and aminopyrine metabolism by rat liver $9,000 \times g$ supernatants. Homogenates of livers from male

TABLE 1
Effect of dietary thiamine consumption on liver and body weights

Sex and Diet	n ^a	Liver Weight	Body Weight	Liver Weight/Body Weight X 100
		$\bar{x} \pm S.E.$	$\bar{x} \pm S.E.$	
Male				
HT	26	0.60 \pm 0.20	144.0 \pm 3.0	4.64 \pm 0.10
TD	27	0.59 \pm 0.16 ^b	99.8 \pm 3.4 ^b	4.17 \pm 0.06 ^b
Female				
HT	24	5.84 \pm 0.25	184.1 \pm 3.2	4.33 \pm 0.13
TD	23	5.96 \pm 0.36 ^b	98.9 \pm 4.0 ^b	4.30 \pm 0.13

^a n represents number of animals.

^b P < .01.

and female rats fed the HT diet for 21 days metabolized aniline at approximately one-half the rate of those fed the TD diet. Liver homogenates from male and female rats fed the HT diet metabolized soxasolamine at rates 0.75 and 0.68 times those of rats fed the TD diet. Aminopyrine metabolism was significantly depressed only in male rats fed the HT diet. There was no significant difference in the *in vitro* metabolic rate of hexobarbital by either sex as a consequence of diet. It should be noted, however, that rat liver homogenates from females fed both diets metabolized hexobarbital, aminopyrine, aniline and soxasolamine at significantly slower rates than those from males. Similar relationships existed when the metabolic rates were expressed as micromoles metabolized per 100 g of body weight per hour. Although body and liver weights were significantly greater in rats fed the HT diet, aniline metabolism *per rat* was not significantly higher in the males and was lower

in the females than in the thiamine-deficient rats. Females fed the TD diet also metabolized soxasolamine (micromoles per rat per hour) at a rate similar to their counterparts receiving the HT diet. When calculated *per rat*, males fed the TD diet metabolized soxasolamine, hexobarbital and aminopyrine at rates less than those fed the HT diet, whereas females metabolized hexobarbital and aminopyrine less rapidly. V_{max} (calculated on

TABLE 2
Effect of diet on the *in vitro* metabolism of aniline, soxasolamine, hexobarbital and aminopyrine (rat liver 9000 X g supernatants)

Substrate and Sex	Diet	n ^a	Metabolism per Gram of Liver per Hour	Metabolism Rat per H
			$\mu\text{mol} \pm S.E.$	$\mu\text{mol} \pm S.E.$
Aniline				
M	HT	12	0.781 \pm 0.041	8.31 \pm 0.39
	TD	12	1.439 \pm 0.105 ^b	4.17 \pm 0.47
F	HT	8	0.492 \pm 0.030	3.28 \pm 0.13
	TD	8	0.994 \pm 0.062 ^b	8.60 \pm 0.51
Soxasolamine				
M	HT	11	1.809 \pm 0.136	12.66 \pm 0.91
	TD	11	2.516 \pm 0.117 ^b	7.15 \pm 0.35 ^b
F	HT	9	1.360 \pm 0.082	6.00 \pm 0.42
	TD	8	2.074 \pm 0.127 ^b	8.11 \pm 0.39
Hexobarbital				
M	HT	8	1.77 \pm 0.10	30.80 \pm 1.75
	TD	7	1.61 \pm 0.21	17.12 \pm 2.32 ^b
F	HT	8	1.76 \pm 0.01	12.08 \pm 0.68
	TD	8	1.80 \pm 0.01	8.90 \pm 0.02 ^b
Aminopyrine				
M	HT	9	0.439 \pm 0.22	2.81 \pm 0.22
	TD	9	0.632 \pm 0.635 ^b	1.84 \pm 0.13 ^b
F	HT	7	0.133 \pm 0.009	0.39 \pm 0.03
	TD	7	0.115 \pm 0.006	0.34 \pm 0.03 ^b

^a n represents number of animals.

^b P < .01 that differences between the two diets are due to chance.

^c P < .05

TABLE 3
Effect of dietary thiamine on the apparent K_m and V_{max} for aniline hydroxylase and hexobarbital oxidase

Diet	n ^a	V_{max}^b		K_m^b	
		Aniline	Hexobarbital	Aniline	Hexobarbital
		$\mu\text{mol/mg protein/hr} \pm S.E.$		$\text{mM} \pm S.E.$	
HT	8	7.800 \pm 0.136 ^c	142.80 \pm 13.95 ^c	0.132 \pm 0.010 ^c	0.555 \pm 0.102 ^c
TD	8	13.853 \pm 0.349 ^d	183.68 \pm 6.27 ^d	0.094 \pm 0.010 ^c	0.617 \pm 0.040 ^c
Lab chow	8	12.202 \pm 0.561 ^d	175.16 \pm 6.11 ^d	0.130 \pm 0.022 ^c	0.664 \pm 0.041 ^c

^a n represents number of animals.

^b Values represent those calculated from four points on the Lineweaver-Burk plots. Values without common superscripts differ significantly.

TABLE 4

Hexobarbital sleeping times and durations of somasolamine paralysis as affected by dietary thiamine

Sex and Diet	Sleeping Time	Duration of Paralysis
	min \pm S.E.	min \pm S.E.
Male		
HT	28 \pm 2 (11) ^a	192 \pm 15 (8)
TD	29 \pm 2 (11)	111 \pm 12 ^b (8)
Female		
HT	105 \pm 6 (10)	257 \pm 34 (8)
TD	59 \pm 3 ^b (10)	130 \pm 29 ^b (8)

^a Numbers in parentheses indicate number of animals.

^b $P < .01$.

the basis of micromoles metabolized per gram of liver or per milligram of microsomal protein) for hexobarbital was not altered significantly from lab chow controls in male rats fed the TD or HT diets. V_{max} for aniline was significantly less in male rats fed the HT diet than in male rats fed lab chow or TD diets ($P < .01$) (table 3). The K_m for aniline and hexobarbital of rat liver from TD- or HT-fed animals was not significantly different from that of liver from rats fed lab chow.

Hexobarbital sleeping time and duration of somasolamine paralysis. Hexobarbital sleeping times are shown in table 4. Although animals on the TD diet were thiamine deficient (as evidenced by body weight 65% of HT rats), there was no difference in hexobarbital sleeping times for male rats. In females, however, the sleeping time for TD animals was about half that of HT rats. On both diets, females slept longer than males: twice as long as males on the TD diet, and almost four times as long on the HT diet.

Duration of somasolamine paralysis in male and female rats fed the HT diet was approximately twice as long as those fed the TD diet.

Glucose-6-phosphate dehydrogenase (G-6-PD) and 6-phosphogluconate dehydrogenase (6-PGD) activities. Table 5 shows the variation as a consequence of diet in G-6-PD and 6-PGD activities of the 105,000 \times g supernatant fraction from male rat liver preparations. Significant elevations in 6-PGD activity (1.42-fold), and G-6-PD activity (2.23-fold) were seen in animals

fed the HT diet compared to those fed the TD diet.

Effect of soluble fraction components on aniline metabolism. The results obtained with a cross-over technique on male rat liver preparations are shown in table 6. In this technique, the 105,000 \times g microsomal pellet of an animal fed the TD diet was resuspended in the 105,000 \times g supernatant of an animal fed the HT diet. The opposite combination was also employed. The results show that regardless of supernatant fraction present, microsomes from rats receiving the HT diet metabolize aniline at a significantly slower rate than microsomes obtained from rats fed the TD diet. The lack of effect of soluble fraction components was verified by measuring aniline metabolism in the presence of increasing increments of NADP (0.2–1.2 mg added per incubation flask), NADPH (0–4 mmol added per incubation mixture) and G-6-PD (0–1.5 enzyme units added per incubation mixture). Neither of these components enhanced aniline metabolism by microsomes from rats on the HT diet to any greater extent than those from rats on the TD diet.

TABLE 5

Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities of the 105,000 \times g supernatant fractions of rat liver homogenates

Diet	G-6-PD	6-PGD
	$\mu\text{mol NADP reduced/g liver/min} \pm \text{S.E.}$	
HT (10) ^a	97.78 \pm 12.44	32.16 \pm 3.62
TD (10)	27.79 \pm 7.51 ^b	22.73 \pm 2.08 ^b

^a Numbers in parentheses indicate number of animals per group.

^b $P < .01$.

TABLE 6

Rate of aniline metabolism by liver microsomes from rats receiving thiamine-deficient and high thiamine diets (cross-over technique)

Combination ^a	Metabolism
	$\mu\text{mol/g liver/hr} \pm \text{S.E.}$
HT diet microsomes with TD diet supernatant	0.824 \pm 0.048
TD diet microsomes with HT diet supernatant	1.537 \pm 0.130 ^b

^a Eight animals per group.

^b $P < .01$.

TABLE 7

Esterase activity of liver fractions from rats fed thiamine-deficient and high thiamine diets

Sex and Diet ^a	9,000 X g Supernatant ^b	100,000 X g Supernatant ^b	Microsomal Fraction ^b	9,000 X g Supernatant ^c
Male				
HT	643.71 ± 24.59	123.55 ± 9.97	550.29 ± 23.51	
TD	578.65 ± 36.41	118.71 ± 10.47	453.35 ± 32.19 ^d	
Female				
HT	801.26 ± 36.23	95.81 ± 2.12	611.32 ± 37.42	21.57 ± 1.80
TD	736.55 ± 34.78	96.47 ± 4.62	572.65 ± 36.93	16.29 ± 1.00 ^d

^a Nine animals per group.^b Micromoles ± S.E. of α -naphthol acetate metabolized per minute per gram of liver.^c Micromoles ± S.E. of acetylcholine perchlorate metabolized per minute per gram of liver.^d $P < .05$.

Esterase activities. Aliesterase activity as measured by α -naphthol acetate hydrolysis by fractions of male and female rat liver is shown in table 7. More activity was found in the microsomes of rats fed the HT diet, although this was significant only in the males.

Acetylcholinesterase activity was determined in female rat liver 9,000 X g preparations by measuring their ability to hydrolyze acetylcholine perchlorate. As shown in table 7, animals fed the HT diet exhibited significantly greater activity than those receiving the TD diet.

Microsomal protein, cytochrome b_5 , and cytochrome P-450 content. Microsomal protein content was not significantly altered by the test diet in either sex. The cytochrome P-450 and b_5 contents of microsomes of rats fed the HT diet were significantly less than those from rats fed the TD diet for both sexes (table 8).

TABLE 8

Microsomal protein, cytochrome b_5 , and cytochrome P-450 concentrations in livers from rats fed thiamine-deficient and high thiamine diets

Sex and Diet	n ^a	Microsomal Protein	Cytochrome b_5	Cytochrome P-450
		mg/g liver ± S.E.	nmol/mg protein ± S.E.	
Male				
HT	6	34.0 ± 1.2	0.144 ± 0.001	0.300 ± 0.001
TD	6	33.0 ± 2.0	0.303 ± 0.010 ^b	0.402 ± 0.005 ^b
Female				
HT	6	37.5 ± 1.0	0.333 ± 0.014	0.308 ± 0.017
TD	7	38.4 ± 0.6	0.337 ± 0.012 ^b	0.410 ± 0.023 ^b

^a n represents number of animals per group.^b $P < .01$.

TABLE 9

NADPH-cytochrome c reductase activity of microsomes from male rats fed thiamine-deficient or high thiamine diets

Diet ^a	Cytochrome c Reduced
	nmol/mg protein/hr
HT	22.97 ± 0.04 ^b
TD	40.51 ± 3.03 ^c

^a Ten animals per group.^b Values represent means ± S.E.^c $P < .01$.

NADPH-cytochrome c reductase activity. Dietary effect on male rat liver NADPH-cytochrome c reductase activity is shown in table 9. Animals fed the HT diet had approximately 57% of the NADPH-cytochrome c reductase activity of those fed the TD diet.

Discussion. The levels of dietary components such as carbohydrate and protein individually have been reported to alter drug metabolism and to produce changes in cytochrome P-450 levels. However, since the diets in these studies were identical except for thiamine content, differences observed must be due to the thiamine or to the total quantity of diet consumed. Rats fed the thiamine-deficient diet for 21 days uniformly developed beriberi, whereas those fed the high thiamine diet had growth curves and appearance not unlike those of rats fed a standard laboratory chow diet (Wade *et al.*, 1960b).

The test diets produced changes in body weight to a different degree in the two sexes. With body weight as the criterion, the male

appears to be more susceptible to changes in thiamine levels than the female. A similar relationship is seen in the liver weights of each sex. Liver weight as a percentage of body weight for male rats also showed a greater difference as a result of the two diets than that of the females. It was higher in male rats fed the HT diet, whereas no difference was found between treatments in females.

Aniline, soxasolamine, aminopyrine and hexobarbital metabolic rates were estimated *in vitro* to determine their susceptibility to alteration by the HT and TD diets in both male and female rats. The HT diet significantly depressed aniline and someolamine metabolism of both males and females, whereas aminopyrine metabolism was depressed in male rats but was not significantly changed in females. Hexobarbital metabolic rate was not altered by diet in either sex. The metabolic rates of all substrates tested were consistently higher in male rats than in females. Thus, the possibility that thiamine exerted its effects on drug metabolism by altering sex hormone levels is minimal. Although thiamine deficiency produced effects on liver and body weights and aniline metabolism similar to those produced by starvation (Kato and Gillette, 1965), it appears that the effects on drug metabolism reported herein are not due solely to starvation—starvation for three days decreased hexobarbital and aminopyrine metabolism in males and increased both in females whereas soxasolamine metabolism in either sex was not altered. Neither V_{max} nor K_m for aniline was altered significantly in the rats fed the TD diet when compared to those fed lab chow.

Since V_{max} but not K_m for aniline was depressed in the HT-fed male rats when compared to rats fed lab chow, it is assumed that the quantity of enzyme responsible for aniline hydroxylation is decreased but the characteristics of the enzyme are qualitatively unaltered. Hexobarbital metabolism on the other hand was not significantly altered by the HT or TD diet when compared to lab chow controls. Since the level of cytochrome P-450 and the activity of NADPH-cytochrome c reductase were reduced in the rats fed the HT diet, it does not appear that either of these components of the mixed function oxygenase system of rat liver are rate limiting in the case of hexobarbital oxidase, but that either may

be rate limiting in the case of aniline hydroxylation.

In order to verify the alterations of *in vitro* metabolic rates, *in vivo* sleep and paralysis times were determined for hexobarbital and soxasolamine, respectively. As anticipated, hexobarbital sleeping time in the male was not significantly altered by varying thiamine levels. However, female rats fed the HT diet slept significantly longer than those fed the TD diet. This aberration is unexplainable on the basis of the rate of *in vitro* hepatic metabolism of hexobarbital, and therefore, other possibilities such as changes in the central nervous system which alter distribution of the drug to active sites or which increase the sensitivity of these central nervous system sites to the hypnotic should be considered. The fact that females fed the deficient diet slept twice as long as their male counterparts was anticipated from *in vitro* metabolic rates.

The duration of soxasolamine paralysis was shorter for both male and female rats on the TD diet than for those on the HT diet. This relationship can be explained on the basis of metabolic rates since liver homogenates from both male and female rats on the TD diets metabolized soxasolamine faster than those from rats fed the HT diet. The effect of starvation can be ruled out as a contributing factor to this since Kato and Takanaka (1967) reported that the duration of soxasolamine paralysis is increased in fasted rats.

The generating system for NADPH needed for microsomal electron transfer reactions associated with the drug-metabolizing enzyme is located in the soluble liver fraction. G-6-PD is a vital component of this generating system. When the effects of each diet upon G-6-PD and 6-PGD activities were examined, it was found that the livers of rats fed the HT diet had increased G-6-PD activity and 6-PGD activity in comparison to those fed the TD diet. Since aniline, aminopyrine and soxasolamine metabolism is greater in thiamine-deficient animals, it appears that G-6-PD activity of the supernatant is not a limiting factor in their metabolism.

To determine whether other components of the soluble fraction were responsible for these differences, a cross-over technique was employed. Microsomal pellets of livers from rats on each diet were prepared. The microsomes recovered

the soluble fraction from livers of rats fed the HT diet, and HT microsomes were resuspended in the TD soluble fraction. Controls were established in which the microsomes from rats on the HT and TD diets were resuspended in their homologous supernatants. The preparations containing the microsomes produced by a TD diet, regardless of soluble fraction present, metabolized aniline at a faster rate than microsomes produced by the HT diet. From these experiments it can be assumed that alterations occurring in the soluble fraction as a consequence of diet do not significantly affect drug metabolic rate and that the limiting alterations produced by the diet are located in the microsomal fraction of the liver cells. These findings also demonstrate that the occurrence of a drug-metabolizing repressor in the soluble fraction of rats on high thiamine diets is remote.

The possibility of the test diets producing a variation of *allosterase* and *acetylcholinesterase* activities was investigated. Neither enzyme requires added co-factors. *Allosterase* activity as determined by the hydrolysis of α -naphthol acetate was higher for the HT than the TD diet in both male and female microsomal liver preparations. *Acetylcholinesterase* activity as determined by the hydrolysis of acetylcholine perchlorate was higher for the HT diet than the TD diet in female preparations. These are consistent with the conclusion that the variations produced by the diets were not due to co-factor alteration.

Further investigation was then initiated to determine the apparent alterations in microsomal protein, cytochrome P-450 and cytochrome *b*₅ contents and NADPH-cytochrome *c* reductase activity. Microsomal protein content is an indication of total enzyme protein. Male rats showed no variation in microsomal protein content, whereas females demonstrated a slightly higher protein content when fed the HT diet than when fed the TD diet. It can therefore be assumed that the variations in metabolic activity caused by the TD and HT diets were not a consequence of altering total enzyme protein levels although this does not rule out the possibility of an alteration in the level of specific enzyme protein.

Both microsomal cytochrome *b*₅ and microsomal cytochrome P-450 levels were significantly

compared to rats fed the TD diet. Cytochrome P-450 levels were depressed 28% in males and 82% in females; whereas *b*₅ content was decreased 52% in males and 38% in females.

NADPH-cytochrome *c* reductase, a hepatic microsomal enzyme (Phillips and Langdon, 1962) necessary for electron transport to cytochrome P-450, likewise had decreased activity in rats fed the HT diet as compared to those receiving the TD diet. Thus, dietary thiamine-induced alterations in hepatic microsomal aniline hydroxylase activity may be partially or wholly due to altered levels of cytochrome *b*₅, cytochrome P-450 and the activity of NADPH-cytochrome *c* reductase. However, it does not appear that either of these components are rate limiting in the case of hexobarbital oxidase. If we assume that dietary thiamine is producing a single effect, we must either assume that this effect is upon the synthesis or maintenance of the specific enzymes involved in various metabolic pathways or that the effect is upon the electron transport system and that this system is rate limiting only for specific pathways.

The possibility that thiamine works indirectly through some other mechanism to produce these alterations must not be overlooked. Animals on the HT diet consumed more diet which was high in carbohydrate. Hara *et al.* (1968) have reported a decrease in *D*-glucose intestinal transport as a result of thiamine deficiency. In this light the observed metabolic alterations may have been a result of varying carbohydrate consumption.

CONCLUSIONS. Aniline and soxasolamine metabolic rates are significantly depressed in both sexes by high dietary thiamine content. *V*_{max} for aniline hydroxylase in the male rat was significantly lower in the rats receiving thiamine than either TD rats or lab chow controls. Amino-pyrene metabolic rate is depressed by high dietary thiamine consumption in the male rat, but not significantly altered in the female rat. Hexobarbital metabolic rate is not significantly altered in male or female rats. When compared to rats fed lab chow, *K*_m for aniline or hexobarbital is not altered in male rats as a result of varying thiamine consumption. α -Naphthol acetate hydrolysis is significantly increased in male rats fed a high thiamine diet. Varying dietary thiamine consumption produced its effect upon the

metabolism through factor(s) within the microsomal fraction of the liver and not in the soluble fraction. Varying thiamine consumption does not alter total hepatic microsomal enzyme protein. Compared to rats fed a thiamine deficient diet, high thiamine consumption decreased hepatic microsomal cytochrome P-450 and cytochrome b₅ contents and hepatic microsomal NADPH-cytochrome c reductase activity. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase activities are depressed by thiamine deprivation.

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A. Comparison of the Acute Toxicity of Two Forms of Thiamine.*

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Recently there has become available for investigational use a new form of vitamin B₁,

thiamine mononitrate. Considering the wide therapeutic use and the parenteral toxicity of thiamine hydrochloride,¹⁻¹³ it was thought

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¹ Steinberg, C. L., *Am. J. Digestive Dis.*, 1938, 5, 680.

ACUTE TOXICITY OF TWO FORMS OF THIAMINE

TABLE I.
Properties of Two Forms of Thiamine.

Property	Thiamine Hydrochloride	Thiamin Mononitrate
Melting point	245-248°C	196-200°C dec.
Molecular wt	337.26	327.36
Units per mg	333	343
Solubility	100 g per 100 g water	2.7 g per 100 g of water

advisable to compare the hydrochloride and the mononitrate in regard to their toxicity in animals.

Comparison of the structural formulas of both forms of thiamine shows where the changes in substituent groups have been made.

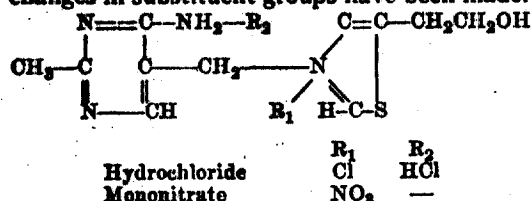


Table I shows the chemical and physical characteristics of both compounds.¹⁴⁻¹⁵ From these data one can conclude that, although the 2 forms are approximately equal in potency, their solubility characteristics would favor the use of the hydrochloride when large doses are to be administered parenterally. However, the solubility of the mononitrate increases to 18.5 g per 100 cc when the pH of the solutions is adjusted to 4.0. Aqueous solutions at this pH are stable for one year,¹⁴ while aqueous solutions of the hydrochloride

at pH 2.7 to 3.0 begin to show a loss of potency in about 6 months.¹⁶

Acute toxicity studies of thiamine hydrochloride have shown that, although large oral doses were toxic, these doses were much larger than those used therapeutically.¹⁷⁻²⁰ However, the same is not true of parenterally administered doses of the drug. Early studies indicated toxic symptoms both in animals and humans^{1,2,18-20} and recently Haley and Flesher²¹ found that rabbits developed symptoms of toxicity after intravenous injections of 200 to 300 mg per animal. This work has been extended to dogs by Smith *et al.*²² who have reported similar results.

Experimental. Acute toxicity of the hydrochloride was determined by intraperitoneal injection in mice and intravenous injection in rabbits and of the mononitrate by intraperitoneal and intravenous injection in mice and intravenous injection in rabbits. In the mouse experiments, a total of 150 animals weighing 22-42 g were used and the concentration of the drugs was 50 mg/cc. The dosage ranged from 0.04 to 0.07 cc intravenously and from 0.17 to 0.32 cc intraperitoneally. Death occurred within 5 minutes after intraperitoneal and within 30 seconds after intravenous injection in mice. In the rabbit experiments

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- ²² Smith, J. A., Foa, P. P., and Weinstein, H. R., *Fed. Proc.*, 1947, 6, 204.

TABLE II.
Toxic Doses of Two Forms of Thiamine.

Animal	Form	Route of administration	Dose mg/kg	Mortality ratio	LD ₅₀ mg/kg	Standard error
Mouse	Mononitrate	Intravenous	80	0/5	84.24	±1.14
			84	4/5		
			86	3/5		
			88	3/5		
			90	3/5		
			92	5/5		
	"	Intraperitoneal	380	1/5	387.3	±1.65
			385	3/5		
			390	2/5		
			395	4/5		
			400	3/5		
	Hydrochloride	"	310	3/5	329.8	±3.93
			320	3/5		
			330	3/5		
			335	4/5		
			340	4/5		
			350	4/5		
Rabbit	Mononitrate	Intravenous	Animal wt, kg	Total dose, mg	Intrav. lethal dose, mg/kg	Avg intrav. lethal dose, mg/kg
			3.66	500	136.61	112.58
			4.30	475	110.46	
			3.22	375	116.46	
			4.44	437.5	98.53	
			4.66	470	100.85	
	Hydrochloride	"	1.704	180	105.63	117.45
			1.818	220	121.01	
			1.591	200	125.70	

the dosage was 1 cc (50 mg/cc) per minute until death, which occurred within 10 minutes. The rabbits used weighed 3.22 to 4.44 kg. Table II gives the mortality figures for mice including the LD₅₀ which is the dose calculated to kill 50 per cent of the mice, according to the method of Miller and Tainter;²³ Table II also lists the intravenous dose required to kill all of the rabbits injected.

The symptoms of toxicity observed were: restlessness, labored respiration, vasodilatation, cyanosis, muscular twitching, clonic convulsions and death by respiratory paralysis. This respiratory paralysis was of central origin because electrical stimulation of both the muscle of the diaphragm and the phrenic nerve showed that the muscle was still capable of contraction. Visual signs of anoxia were a gradually deepening bluish coloration of the

ears and all other body areas where the fur was thin enough to permit direct observation of the skin. In all animals cardiac arrhythmias were seen upon opening the thoracic cage. Auricular/ventricular rates of from 2:1 to 5:1 were common.

In conjunction with these toxicity studies more than 100 unanesthetized rabbits, weighing between 3.11 and 5.33 kg, were injected intravenously with solutions of thiamine hydrochloride ranging from 10 to 100 mg/cc. This routine testing over the period of one year showed that, although fatalities were seldom observed, clonic convulsions were produced in 80% of the animals when the total dose was above 300 mg per animal. These convulsions usually occurred after the animal had been returned to its cage. No visual signs of anoxia, cyanosis of the blood in the ear veins or of the skin in the scrotal region, were seen. However, no blood samples were

²³ Miller, L. C., and Tainter, M. L., *Proc. Soc. Exp. Biol. and Med.*, 1944, 57, 261.

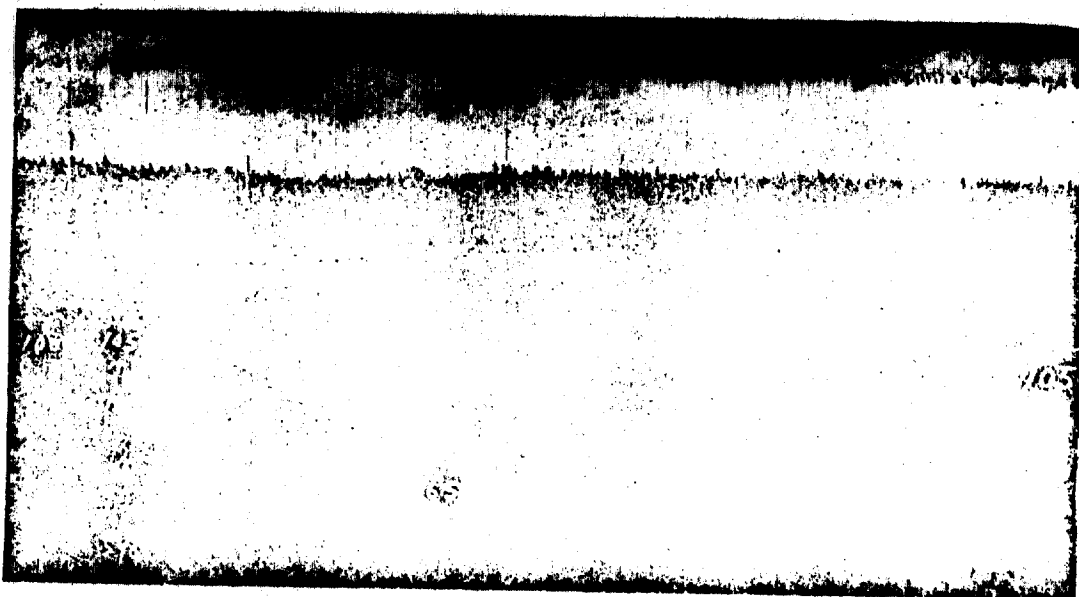


FIG. 1.
Top, Respiration. Bottom, Blood Pressure.

obtained for the determination of oxygen content so that partial anoxia cannot be entirely ruled out as a causative factor in the convulsions.

Inasmuch as Molitor²⁰ has shown that thiamine has a pronounced effect upon respiration and little effect upon the blood pressure of the dog, it was decided that a study of this phenomenon should be undertaken. Six rabbits, weighing 3.66 to 5.22 kg were anesthetized with 20 mg/kg of sodium pentobarbital intravenously and 3.5 cc/kg of 20% urethane intraperitoneally and prepared for recording of blood pressure via the carotid artery and respiration by cannulation of the trachea. Respiration was recorded with the Haley respirometer²⁴ and blood pressure with a mercury manometer. The two forms of thiamine in a concentration of 50 mg/cc in normal saline were injected at a fixed dose of 120 mg alternately. As the rate of injection partially determines the effect on the animal, 2 injection rates were employed: slow (1 mg/second) and fast (12 mg/second). Further, in order to rule out the effect of pH, 2 rabbits were injected with the mononitrate at pH 6.8 and 4 at pH 0.9. The hydrochloride was always at pH 2.7. Fig. 1 shows a typical

record of a slow injection and Fig. 2 is typical record of a fast injection.

The results of this work show that slow injections of either form of thiamine had very little effect on respiration but caused a fall in blood pressure averaging 36 mm of mercury. This fall was gradual, requiring 114 seconds to be completed and the recovery required 219 seconds to reach the previous normal level. Fast intravenous injections had a more pronounced effect on respiration, decreasing both the rate and the depth. There was also a blood pressure fall averaging 23 mm of mercury.

Discussion. From the results of the acute toxicity determinations given in Table II there appears to be little difference in the toxic doses of either form of thiamine. Further, Molitor²⁵ has found that the mouse intravenous toxicity of the hydrochloride is 85 mg/kg which agrees with the 84.24 mg/kg figure for the mononitrate.

From the results herein presented as well as those of previous investigators²⁰⁻²² one must conclude that thiamine exerts its principal toxic effect by paralysis of the respiratory center. However, the work of Zaidi²⁶ on the

²⁴ Haley, T. J., *J. Am. Pharm. Assn.*, in press.

²⁵ Molitor, H., personal communication.

²⁶ Zaidi, S. H., *Ind. Med. Gaz.*, 1947, 82, 181.

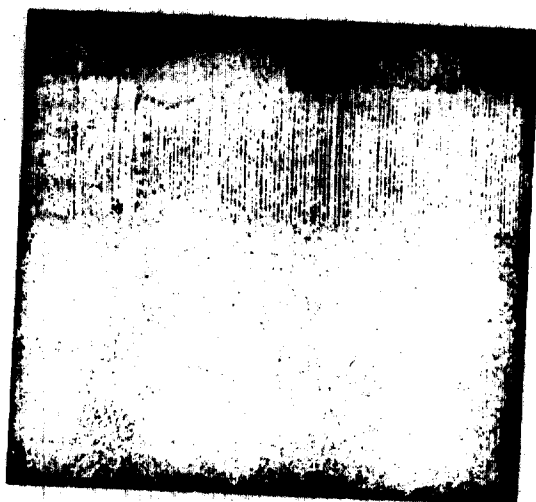


FIG. 2.

Top, Respiration. Bottom, Blood Pressure.

isolated frog heart and Smith *et al.*²² on the isolated turtle heart indicates that thiamine has also a direct toxic effect on the myocardium. Furthermore, irregularities in the electrocardiogram of dogs after the administration of large doses of thiamine²² shows that the mammalian heart is affected.

Smith *et al.*²² found that, even after vago-

tomy or atropinization, there was a prolonged peripheral blood pressure fall. Haimovici and Pick²⁷ reported that thiamine caused vasodilatation which counteracted the vasoconstrictor action of nicotine on the perfused frog hind limb preparation. Thus it is probable that thiamine causes vasodilatation by direct action on the peripheral vascular musculature.

Summary. There is little difference in the lethal dose of either thiamine hydrochloride or mononitrate and the symptoms of toxicity are the same for both forms of the drug. The toxic effects of both forms of the vitamin on respiration and blood pressure are due to their thiamine content and not to the pH of the injected solutions or to the substituent groups on the nitrogen of the thiazole nucleus. In lethal doses either form of the vitamin causes death by a direct paralyzing action on the respiratory center followed by cardiac failure.

The author wishes to thank Merck and Co. for the generous supply of thiamine mononitrate used in this study.

²⁷ Haimovici, H., and Pick, E. P., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 234.

TABLE 1

Patch Tests

Results of patch tests with components and derivatives of thiamine. Water was used diluent. Patch tests were left for 48 hours and read after 48 and 72 hours. 1+ erythema, 3+ erythema, infiltration vesicles.

Substance	Date	Conc.	Reaction
Thiamine	11/4/55	Pure	+
	11/7/55	10%	+++
	11/17/55	1%	+++
		0.1%	+
		0.01%	-
	2/11/56	0.1%	+
Co-Carboxylase	2/13/56	10%	+++
		1%	+++
		0.1%	-
4-methyl-5-(oxyethyl)-thiazole	2/13/56	Pure	-
		10%	-
2-methyl-6-amino-5-brom-methyl-pyrimidine*	2/11/56	Pure	+++
		10%	+++
	2/13/56	10%	-
		1%	-
2-methyl-6-amino-5-amino-methyl-pyrimidine	2/11/56	Pure	-
		10%	-
Sulfathiazol-cream	11/17/55	5%	-
Sulfapyrimidine (Sulfadiazine)	2/13/56	Pure	-

* A primary irritant.

The substances employed for patch testing were kindly supplied by Hoffmann-La Roche Ltd.

reactions. Further patch tests with occupational contactants led to the demonstration of positive reactions to thiamine, even in a 0.1% dilution. The patient had sometimes been employed filling vials with thiamine and had noticed irritation of the skin during this work. She further

supplied the information that she daily took a tablet of vitamin B. This was found to contain mg. of thiamine.

Under conservative treatment the eczema disappeared after a few months. Afterwards the patient changed her occupation and from January

She worked as a domestic servant employed in housework and cleaning without experiencing any further irritation of the skin. She had no contact with thiamine and took no vitamin B tablets. After 6 weeks she returned for a follow-up examination, which disclosed only residual signs of identification of the former patches of eczema.

Experimental provocation: In the period of remission during which the patient continued her work, a sudden flare-up of the eczema was observed twice. On the first occasion it was revealed to the patient, on her own initiative, had resumed work filling thiamine vials.

The second time, the eczema flared up as a result of experimental provocation. In order to determine the effect of ingested thiamine two tablets of 100 mg. thiamine each were administered on the 6th of December 1955. Next day the almost healed patches of eczema were red and itching. An additional 100 mg. of thiamine was administered in a coated tablet. The Sunday she had an acute relapse to the state prior to treatment, but after a week the eruption faded.

A similar relapse occurred 2 months after the eczema had disappeared. At the control examination on the 11th February 1956 supplementary patch tests, scratch- and intracutaneous tests were performed (cf. table 1). Patch tests with thiamine were positive as previously, and a cross-reaction to co-carboxylase was demonstrated. Intracutaneous tests with 0.1 ml. of 0.1% solutions of thiamine and co-carboxylase were negative. Three days later similar intracutaneous tests with 0.1 ml. of 1% solutions and half an hour later with 1% solutions of the same substances were performed (total dose of thiamine co-carboxylase 22 mg.). The four injections all provoked immediate papular response, as is the case in many normal persons.

Between 6 and 10 hours after the injections a perioral area of erythema appeared around the mouth. Shortly after both hands and wrists were similarly involved. Next day the pruritus had subsided, but an intensely red, vesicular dermatitis was found in all the formerly affected areas. The 5 day old patch tests showed no focal reactions, but late papular reactions of 20 by 20 mm., without vesicles, had appeared at the sites of the intracutaneous injections with thiamine 1% and 10%, while the co-carboxylase had given a doubtful reaction.

Under treatment with zinc oxide lotion the

TABLE 2
Reactions to thiamine solutions of different pH. Intradermal tests with 0.05 ml. in 20 patients.

	pH	Wheal, mm	
		Average	Range
Thiamine HCl, 4%	3	11.25	9.5-13.5
Thiamine, 4%	5.6	10.25	7-13.5

dermatitis disappeared with scaling within ten days.

CONTROLS

Consecutive dermatological patients served as controls.

Patch tests with thiamine 50% in water and with co-carboxylase 1% in water were negative in 100 patients. Patch tests with pure methylamino-bromomethyl-pyrimidin (cf. table 1) were done on 34 patients of which 6 showed positive reactions clinically of the primary irritancy type. All had negative reactions to a 10% solution (and to thiamine). Later 122 patients were tested with a 10% solution of the same pyrimidine derivative. One patient showed a positive eczematous reaction to a 10% and a 5% solution, but negative reaction to thiamine. The cause of the positive reaction could not be ascertained.

Intracutaneous tests with thiamine confirmed that this substance normally produces a wheal and a flare. If 5% and 10% solutions are employed such reactions are often accompanied by pseudopods.

Similar reactions may, however, be provoked in many normal persons by intracutaneous tests with 1% acetic acid which has about the same pH as a thiamine solution.

In order to determine whether the whealing effect of thiamine might be due to the low pH of the solutions, the reactions to a 4% solution of thiamine were compared to those of a thiamine solution of pH 5.6. The latter solution was prepared by mixing equal parts of a 2 normal NaOH with an 8% solution of thiamine. Owing to the instability of thiamine at the resulting pH of 5.6 the solution was prepared immediately before use and discarded after 60 minutes. The results (cf. table 2) confirmed that the reactions are due to some specific action of thiamine.

The whealing after intracutaneous injections of thiamine is generally attributed to an enhancing effect upon the cholinergic nervous system (2, 4). In two patients, however, with cho-

linergic urticaria and a verified high sensitivity to cholinergic substances, intracutaneous reactions to thiamine did not show any peculiarities.

Intracutaneous tests with co-carboxylase 1% 0.05 ml. were performed in 30 patients and showed in 25 cases a wheal ranging from 5 to 14 mm. accompanied by a flare up to 40 mm. The flare remained for more than 20 minutes. 5% and 10% solutions provoked similar reactions but left a central necrosis, probably owing to the strongly acid reaction (pH 1.5-1.8).

DISCUSSION

Of special interest in the present case is the relapse of eczema provoked by the ingestion of thiamine. Dalton and Pierce (5) administered thiamine by mouth, in unstated dosage, to ten patients with positive patch tests to aneurin. No "untoward signs or symptoms resulted." In the present case the eczema relapsed after ingestion of a dose of thiamine far above the physiological requirements, but of a size used therapeutically. However, the dose of tolerance could not be determined owing to the reluctance of the patient to permit further tests.

It is particularly striking that a vitamin which normally plays an essential part in cellular metabolism should have antigenic properties. So it might be presumed that the primary allergen is an impurity, possibly a decomposition product, which under occupational conditions of exposure, might occur in sufficient concentration for sensitizing.

Impurities, however, could not explain the hypersensitivity in the present case, where patch tests with pure crystalline preparations from different sources (Merck; la Roche) were all positive.

The decomposition products have not been tested in any of the previously reported cases of hypersensitivity to thiamine. Sensitization to these products might occur since thiamine is unstable at the prevailing pH of the skin surface.

Patch tests with the pyrimidine-component of thiamine pure and in 10% solutions were positive, but repeated tests with 10% and 1% solutions were negative (cf. table 1). As thiamine elicited positive reactions even in 0.1% solutions, it is unlikely that the decomposition products could be the primary allergen.

Although the pyrimidine derivative employed

for the tests is a primary irritant, the reactions provoked by it might be an expression of a cross-sensitization or secondary allergy, provided the pyrimidine part was the antigenic determinant group of the thiamine molecule. This cross-sensitization should, however, have included the amino-methyl-pyrimidine, which was tested but with a negative result. Thus the observed reactions from the bromo-methyl-pyrimidine must have been due to the primary irritant properties of the substance, and it may be concluded that the primary allergen in this case was the whole thiamine molecule.

Considering the chemical structures it is understandable that a cross-reaction to co-carboxylase could be demonstrated. This is, however, of some theoretical interest, as it seems to be the first demonstration of a hypersensitivity to a pure co-enzyme.

SUMMARY

1. A case of occupational dermatitis from thiamine is reported. Relapses of the eczema occurred after ingestion of thiamine (200 mg) and later after intracutaneous injection of 10 mg thiamine.
2. Patch tests with the components indicated that the antigenic determinant was the whole molecule.
3. A secondary allergy to co-carboxylase was demonstrated.

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RELATIVE STABILITY OF THIAMINE MONONITRATE AND THIAMINE CHLORIDE HYDROCHLORIDE IN ENRICHED FLOUR¹

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ABSTRACT

Storage tests were conducted on samples of flour enriched with thiamine mononitrate and thiamine chloride hydrochloride, respectively, under both normal and accelerated conditions. The relative stability of thiamine in enriched flour decreased with increased temperature and flour moisture during storage. However, the stability of thiamine mononitrate in flour was influenced less by the temperature and the moisture conditions of the flour than was the stability of thiamine chloride hydrochloride. For example, enriched flour samples with 14.5% moisture lost 40% and 27%, respectively in thiamine chloride hydrochloride but only 5% or less in thiamine mononitrate during storage for 4 months at 38°C. and room temperature.

The increased stability of thiamine mononitrate in enriched flour is attributed to its relatively low hygroscopicity compared to thiamine chloride hydrochloride.

With the advent of the flour enrichment program, several laboratories studied the stability of the enriching vitamins in flour. It was generally recognized that thiamine was slowly destroyed during the storage of enriched flour, and that the rate of destruction was dependent upon the temperature and moisture content of the flour. Early estimates by the Technical Advisory Committee of the Miller's National Federation place the average loss in thiamine during six months dry storage of enriched flour at 5%. Later estimates tended to place the six months dry storage loss at nearer 15%.

In 1945, a report from the laboratories of Anheuser-Busch, Inc., (1) showed losses of 24% and 46%, respectively, in thiamine chloride hydrochloride during storage of enriched flour samples for six months at 72°F. and 100°F. Although these storage losses were observed for thiamine in samples of flour stored in closed, glass bottles, they were indicative of the losses which could occur during regular commercial flour usage, including storage and transportation.

Federal and state enrichment standards (3) for enriched flour specify that it contain not less than 2.0 mg. and not more than 2.5 mg. of thiamine per pound of flour. In the production of enriched

¹ Manuscript received July 27, 1951. Contribution from the Research Laboratories of Merck and Co., Inc., Rahway, N. J. Presented at the Annual Meeting May, 1951.
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flour, the losses in thiamine encountered during storage and transportation of the product must be taken into account, since the product must meet the specifications of these standards even after prolonged storage and transportation in hot, humid weather. It is general practice to add an extra amount of thiamine to flour to compensate for any losses that may occur during storage and transportation. In general, a small overage in thiamine is ample, since the losses in thiamine are small during the ordinary handling of flour. However, there is some question as to whether the total allowance for thiamine between the maximum and the minimum in the flour standards (0.5 mg. per lb.) would make up for the losses occurring during handling and storage of enriched flour under unusual conditions.

In a study aimed at increasing the stability of thiamine and thereby eliminating the storage losses in flour, Obermeyer and Schoeffel (4) prepared and tested many different salts of thiamine. These investigators found that the mononitrate salt of thiamine was unusually stable during storage in flour mixtures.

The present report summarizes some of the results of studies on the relative stability of thiamine mononitrate and thiamine chloride hydrochloride in flour, and illustrates the influence of temperature and moisture on the stability of thiamine in flour.

Materials and Methods

Three separate storage tests were conducted on various samples of flour enriched with thiamine chloride hydrochloride and thiamine mononitrate, respectively. In the first test, 1 lb. samples representing nine different commercial flours were enriched in the laboratory and stored in paper bags at room temperature for approximately one year. In the second test, 5 lb. samples of commercially enriched flours were stored in cloth bags for about seven months under room conditions. After about 3 months portions of these commercially enriched samples were also stored in a desiccator at about 70% relative humidity to compensate for the excessive moisture loss that occurred under room humidity. In the third test, flour samples were conditioned to the desired moisture levels by storing in desiccators under controlled humidity, enriched, and stored for four months in sealed glass containers.

The thiamine chloride hydrochloride and thiamine mononitrate used in these studies were U.S.P. grade or its equivalent. In all cases the thiamine was blended with riboflavin, niacin, and iron according to the regular flour enrichment mixture formula prior to the addition to flour. The enrichment mixtures were added to flour samples at levels to give 2.0 to 2.5 mg. of thiamine per pound of flour.

Thiamine and flour moisture determinations were conducted by the official (2) procedures at periodic intervals during the storage tests.

Results

The average rates of loss in thiamine for the flour samples enriched in the laboratory with thiamine chloride hydrochloride and thiamine mononitrate respectively and stored for about one year are shown in Fig. 1.

The average flour moisture at the start of the tests was 12% but during the storage it decreased to 9.2%. The storage temperature

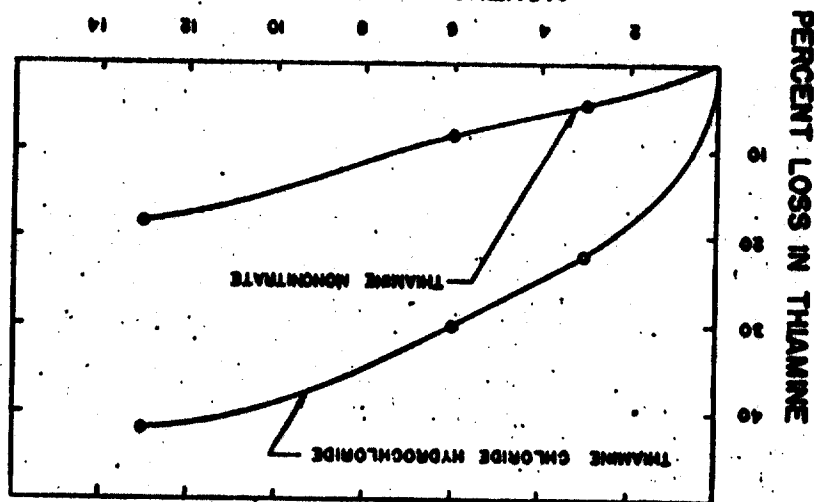


FIG. 1. Relative loss in thiamine during storage of laboratory enriched samples of flour under room conditions.

ranged from a low of 60°F. to a high of 100°F. with an average of about 75°F.

The results of this first storage test show that at the end of 13 months storage the flour samples enriched with thiamine mononitrate

lost only 18% in thiamine compared to a loss of 42% in thiamine for

the samples enriched with thiamine chloride hydrochloride.

The average rates of thiamine loss for the commercially enriched

flour samples are shown in Fig. 2.

Since the humidity of the room was quite low, the flour samples in

this second storage test dried out to less than 10% moisture within a

few weeks. However, the portions of each sample which were stored

in a desiccator at 70% relative humidity absorbed moisture, and at

the end of the test storage the moisture content had increased to 11.7%.

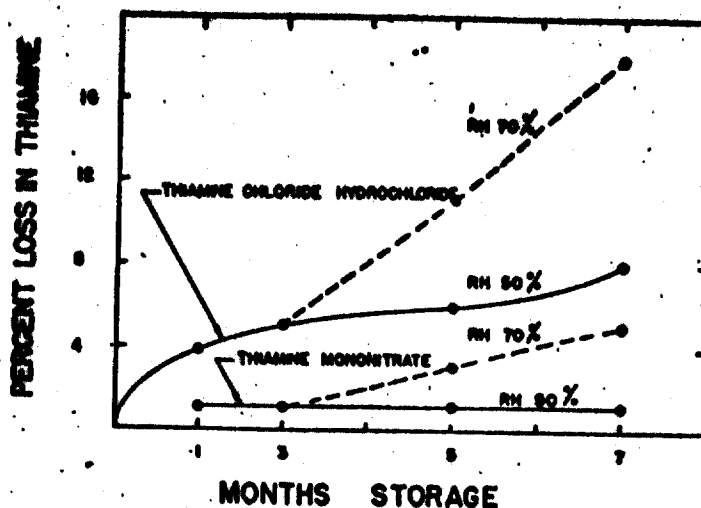


FIG. 2. Relative loss in thiamine during storage of commercial samples of enriched flour at room temperature and 30% and 70% relative humidity.

The curves in Fig. 2 demonstrate the increased rate of loss in thiamine with increased storage humidity (flour moisture). Although thiamine mononitrate is shown to be more stable than thiamine chloride hydrochloride under dry conditions, the difference in the stabilities of the two thiamine salts is even more striking at the higher moisture levels.

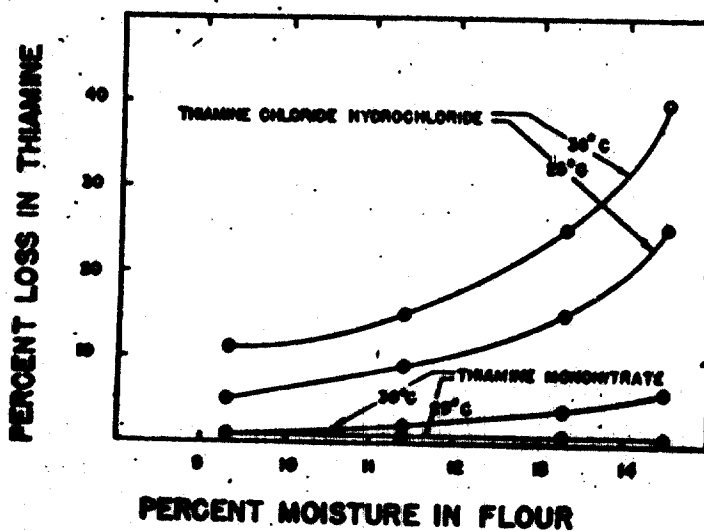


FIG. 3. Effect of flour moisture and storage temperature on the stability of thiamine in flour stored in sealed containers.

Further illustration of the combined effects of increased temperature and flour moisture on the relative stabilities of the two thiamine salts in enriched flour is shown in Fig. 3.

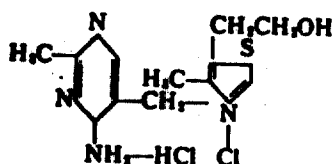
The results of these storage tests show comparatively small effects of higher temperatures and humidities during flour storage on thiamine mononitrate when compared to the effects of these factors on thiamine chloride hydrochloride. It may be concluded then that flour enriched with thiamine mononitrate is much more likely to withstand adverse storage and transportation conditions and still meet the specifications of enrichment standards than flour enriched with thiamine chloride hydrochloride.

Discussion

Crystalline thiamine chloride hydrochloride is generally found to be stable when stored as a solid for extended periods even at elevated temperatures in the presence of air. It is assumed then, that the decomposition of thiamine in flour is dependent upon its solution in water. This assumption is strengthened by the observation that the rate of decomposition of thiamine in flour is increased by increasing the moisture content of the flour.

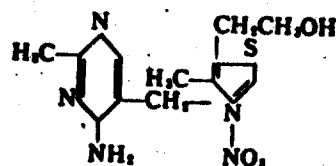
A probable explanation of the relative stability of the mononitrate salt of thiamine in flour can be developed from a comparison of the hygroscopicity and water solubility of the mononitrate salt and the hydrochloride salt. Some of the pertinent physical properties of the two salts are as follows:

Name: Thiamine chloride hydrochloride
Formula:



Molecular weight: 337.3
Water solubility g./ml.: 1
pH saturated solution: 2.5-3.0
Moisture content % (60% R.H.): 4

Thiamine mononitrate



327.3
0.03
6.6-7.0
0.1

Since thiamine mononitrate is less water soluble and less hygroscopic than thiamine chloride hydrochloride, there is a slower rate of solution of the thiamine in the flour moisture, and consequently a slower rate of decomposition.

In connection with the use of thiamine mononitrate as an enriching agent for flour, the following points might be mentioned:

(1) Thiamine mononitrate is a white crystalline solid, and is fully available and active as vitamin B₁ in nutrition.

(2) It meets all requirements under the federal and state Standards of Identity for enriched flour.

(3) It was made commercially available in January, 1950 and has received wide acceptance by the milling industry.

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ON THE ACUTE EFFECT OF SOME DRUGS ON
THE HIGHER NERVOUS ACTIVITY IN MAN.
PART VIII. THIAMINE (20 mg AND 100 mg),
ASCORBIC ACID (500 mg)

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K. DOSTÁLOVÁ, A. ŠIROKÁ

The purpose of this communication is to report on the results obtained from an experimental series which has been designed to investigate the acute effect of vitamins on the higher nervous activity in man. The tested drugs were administered to the persons under investigation in single peroral doses: vitamin B₁ (thiamine - 20 mg and 100 mg), vitamin C (ascorbic acid - 500 mg), and placebo.

METHOD

The experiments were carried out under standard conditions on 16 healthy and unfatigued volunteers - university students (8 females and 8 males, age ranging from 17-18 years). The experiments were performed during the morning and forenoon hours by adopting the method of artificial conditioned speech connections (the so-called laboratory language method). The method made use of and the experimental conditions have already been described in one of our earlier papers.^{8, 9, 10}

The association: "object seen - laboratory word heard" were applied. The principle of the "double blind" experiment was maintained. During the whole experiment, the investigator proceeded only on the basis of the code system.

The criteria for the evaluation of the results were the number of necessary repetitions for mastering the given task, then the number of correct responses

Source of variation	Sum of squares	Degree of freedom	Mean square	Test F	Significance P
Persons	229	15	15.26	2.08	$P < 0.05$
Weeks	158	3	52.67	7.18	$P < 0.01$
Drugs	41	3	14.67	2.09	$P > 0.05$
Sets of words	2	3	0.67	0.09	$P > 0.05$
Sets of objects	13	3	4.33	0.59	$P > 0.05$
Day \times time of day	11	3	3.67	0.59	$P > 0.05$
Residuum	242	33	7.33	—	—
Total	699	63	—	—	—

Tab. 1. Analysis of variance - number of necessary repetitions (NNR) in experiments before administration of the drugs.

Persons	621	15	41.40	2.87	$P < 0.01$
Weeks	85	3	28.33	1.96	$P > 0.05$
Drugs	38	3	12.67	0.88	$P > 0.05$
Sets of words	28	3	9.33	0.65	$P > 0.05$
Sets of objects	127	3	42.33	2.91	$P < 0.05$
Day \times time of day	39	3	13.00	0.99	$P > 0.05$
Residuum	476	33	14.42	—	—
Total	1,414	63	—	—	—

Tab. 2. Analysis of variance - number of necessary repetitions (NNR) in experiments 1 hour after administration of the drugs.

Persons	417	15	29.80	2.04	$P < 0.05$
Weeks	37	3	12.33	0.85	$P > 0.05$
Drugs	37	3	12.33	0.85	$P > 0.05$
Sets of words	57	3	19.00	1.30	$P > 0.05$
Sets of objects	45	3	15.00	1.03	$P > 0.05$
Day \times time of day	26	3	8.67	0.60	$P > 0.05$
Residuum	481	33	14.57	—	—
Total	1,130	63	—	—	—

Tab. 3. Analysis of variance - number of necessary repetitions (NNR) in experiments two hours after administration of the drugs.

Persons	1,704	15	113.60	5.97	$P < 0.01$
Weeks	813	3	271.00	14.24	$P < 0.01$
Drugs	340	3	113.33	5.95	$P < 0.01$
Sets of words	8	3	2.67	0.14	$P > 0.05$
Sets of objects	217	3	72.33	3.80	$P < 0.05$
Day \times time of day	114	3	38.00	2.00	$P > 0.05$
Residuum	628	33	19.03	—	—
Total	3,824	63	—	—	—

Tab. 4. Analysis of variance - number of correct responses (NCR) in experiments before administration of the drugs.

Source of variation	Sum of squares	Degree of freedom	Mean square	F-test	Significance level P
Persons	2,392	15	159.46	1.593	P > 0.05
Weeks	31	3	11.33	0.288	P > 0.05
Drugs	117	3	39.00	0.56	P > 0.05
Sets of words	130	3	43.33	1.13	P > 0.05
Sets of objects	430	3	143.00	3.72	P > 0.05
Day \times time of day	27	3	9.00	0.22	P > 0.05
Residuum	1,339	33	40.57		
Total	4,542	63			

Tab. 5. Analysis of variance - number of correct responses (NCR) in experiment 1 one hour after administration of the drugs.

Persons	1,324	15	88.27	2.71	P > 0.05
Weeks	38	3	12.67	0.39	P > 0.05
Drugs	166	3	55.33	1.76	P > 0.05
Sets of words	344	3	114.67	3.48	P > 0.05
Sets of objects	33	3	11.00	0.34	P > 0.05
Day \times time of day	278	3	92.67	2.84	P > 0.05
Residuum	1,076	33	32.61		
Total	3,226	63			

Tab. 6. Analysis of variance - number of correct responses (NCR) in experiment two hours after administration of the drugs.

Persons	0.3927	15	0.026 180	2.19	P > 0.05
Weeks	0.1203	3	0.040 100	3.35	P > 0.05
Drugs	0.0156	3	0.005 200	0.43	P > 0.05
Sets of words	0.0778	3	0.025 933	2.17	P > 0.05
Sets of objects	0.0122	3	0.014 067	1.17	P > 0.05
Day \times time of day	0.0118	3	0.004 933	0.41	P > 0.05
Residuum	0.3961	33	0.011 973		
Total	1.3585	63			

Tab. 7. Analysis of variance - frequency of responses (FR) in experiments before administration of the drugs.

Persons	0.6140	15	0.040 933	4.91	P > 0.05
Weeks	0.0994	3	0.033 133	3.97	P > 0.05
Drugs	0.0363	3	0.012 100	1.45	P > 0.05
Sets of words	0.0395	3	0.013 167	1.55	P > 0.05
Sets of objects	0.0947	3	0.031 567	3.19	P > 0.05
Day \times time of day	0.0484	3	0.016 133	1.93	P > 0.05
Residuum	0.2754	33	0.008 345		
Total	1.1077	63			

Tab. 8. Analysis of variance - frequency of responses (FR) in experiment 1 one hour after administration of the drugs.

Source of variation	Sum of squares	Degrees of freedom	Mean square	F	Significance P
Persons	0.3953	15	0.026363	3.91	P = 0.01
Weeks	0.0395	3	0.013167	1.95	P = 0.05
Drugs	0.011	3	0.003633	0.52	P = 0.65
Sets of words	0.0384	3	0.012800	1.85	P = 0.05
Sets of objects	0.041	3	0.013633	0.45	P = 0.05
Day \times time of day	0.020	3	0.006666	0.80	P = 0.05
Residuum	0.2859	33	0.008663		
Total	0.8040	63			

Tab. 9. Analysis of variance - frequency of responses (FR) in experiments two hours after administration of the drugs.

	Weeks	I	II	III	IV	s_e
Before administration	NNR	9.3	7.6	6.1	5.2	0.7
	NCR	26.5	31.0	33.5	36.2	1.1
	FR	0.53	0.56	0.59	0.61	0.025
1 hour after administration	NNR	11.1	8.9	7.9	9.4	0.9
	NCR	26.5	27.4	28.5	27.5	1.6
	FR	0.52	0.52	0.56	0.62	0.023
2 hours after administration	NNR	9.3	8.5	8.6	10.4	0.9
	NCR	26.9	27.4	28.8	27.0	1.1
	FR	0.51	0.53	0.56	0.57	0.024

Tab. 10. Average values of characteristics - the effect of weeks.

	Drugs	Vitamin C 0.5 g A	Placebo B	Vitamin B ₁ 100 mg C	Vitamin B ₁ 20 mg D	s_e
Before administration	NNR	7.8	7.9	6.2	6.2	0.7
	NCR	28.6	31.1	34.9	32.7	1.1
	FR	0.56	0.57	0.60	0.59	0.025
1 hour after administration	NNR	9.8	8.1	10.2	9.2	0.9
	NCR	25.4	29.2	27.7	27.5	1.9
	FR	0.52	0.58	0.57	0.56	0.029
2 hours after administration	NNR	8.6	10.0	9.9	8.3	0.9
	NCR	27.9	24.9	28.6	28.9	1.4
	FR	0.55	0.53	0.56	0.54	0.034

Tab. 11. Average values of characteristics - the effect of the drug.

	Sets of laboratory words	α	β	γ	δ	ϵ
Before administration	NNR	6.8	6.9	7.1	7.4	0.7
	NCR	31.1	31.6	31.3	31.2	1.1
	FR	0.61	0.54	0.56	0.52	0.025
1 hour after administration	NNR	8.7	8.9	10.4	9.4	0.9
	NCR	28.2	24.9	27.3	26.4	1.6
	FR	0.56	0.52	0.53	0.57	0.023
2 hours after administration	NNR	10.7	8.6	8.2	9.4	0.9
	NCR	26.4	31.3	27.1	25.8	1.4
	FR	0.54	0.59	0.53	0.53	0.024

Tab. 12. Average values of characteristics - the effect of sets of laboratory words

	Sets of objects	a	b	c	d	ϵ
Before administration	NNR	7.5	6.4	7.4	6.3	0.7
	NCR	29.3	34.4	31.1	32.4	1.1
	FR	0.55	0.62	0.56	0.59	0.025
1 hour after administration	NNR	9.1	10.6	7.1	10.5	0.9
	NCR	26.4	24.5	31.7	27.2	1.6
	FR	0.56	0.55	0.57	0.55	0.023
2 hours after administration	NNR	9.4	10.2	9.2	7.9	0.9
	NCR	28.2	26.6	27.2	28.2	1.4
	FR	0.57	0.55	0.53	0.54	0.024

Tab. 13. Average values of characteristics - the effect of sets of objects

	Time of day	morning	forenoon	ϵ
Before administration	NNR	6.5	7.6	0.5
	NCR	32.9	30.7	0.8
	FR	0.58	0.57	0.018
1 hour after administration	NNR	10.0	8.7	0.9
	NCR	27.2	27.7	1.1
	FR	0.56	0.55	0.015
2 hours after administration	NNR	9.0	9.1	0.6
	NCR	27.8	27.3	1.0
	FR	0.53	0.56	0.017

Tab. 14. Average values of characteristics - the effect of time of day

and the frequency of responses during the first eight repetitions (testing of the active knowledge of the subjects).

The sources of variability were: the different responses obtained from the persons under investigation, the repetitions during each week in succession, the sets of demonstrated objects, the sets of laboratory (artificial, unknown) words designating the objects, the tested drugs, and the time of the day at which the experiments were performed. The results obtained were assessed on the basis of an analysis of variance (Tables 1-9).

An analysis of the power-function of the statistical test was carried out in order to estimate the sensitivity of the method of artificial conditioned speech connections, i. e. the ability to reveal the effect of the studied drugs on the higher nervous activity in man.³

RESULTS

An analysis of the results has been carried out separately for the number of necessary repetitions, the number of correct responses, and the frequency of the responses obtained during the experiments carried out before, and one and two hours after the administration of the drugs.

Number of necessary repetitions

In the first experiment, a different response of the persons under investigation ($F = 2.08$ for 15 and 33 degrees of freedom, further on only as d. f.) and a pronounced improvement in the responses in the course of the weeks in succession ($F = 7.18$ for 3 and 33 d. f.) were observed. The effect of the different levels of the other factors did not reach statistical significance.

One hour after the administration of the drugs, the responses recorded from the individual persons ($F = 2.87$ for 15 and 33 d. f.) and the degree of difficulty in acquiring knowledge of the sets of demonstrated objects ($F = 2.94$ for 3 and 33 d. f.) showed to differ significantly. The effect of the other factors was statistically insignificant. A pronounced impairment appeared, however, in the results obtained after administration of vitamin B₁ (100 mg) and vitamin C (500 mg).

Two hours after the administration of the drugs, differences were only found in the responses of the individual persons ($F = 2.04$ for 15 and 33 d. f.). The best results were observed in experiments carried out after administration of vitamin B₁ (20 mg) and vitamin C (500 mg).

Number of correct responses

In the first experiment, differences in the responses recorded from the individual persons ($F = 5.97$ for 15 and 33 d. f.) a definite improvement in the responses along with the repetition of the experiments, i. e. in the results obtained from each experiment in succession ($F = 14.24$ for 3 and 33 d. f.), and in the degree of difficulty to acquire knowledge of the sets of demonstrated objects ($F = 5.95$ for 3 and 33 d. f.) were observed. The significance of the effect of the "drugs" has already been discussed in one of our previous papers (Part VI).

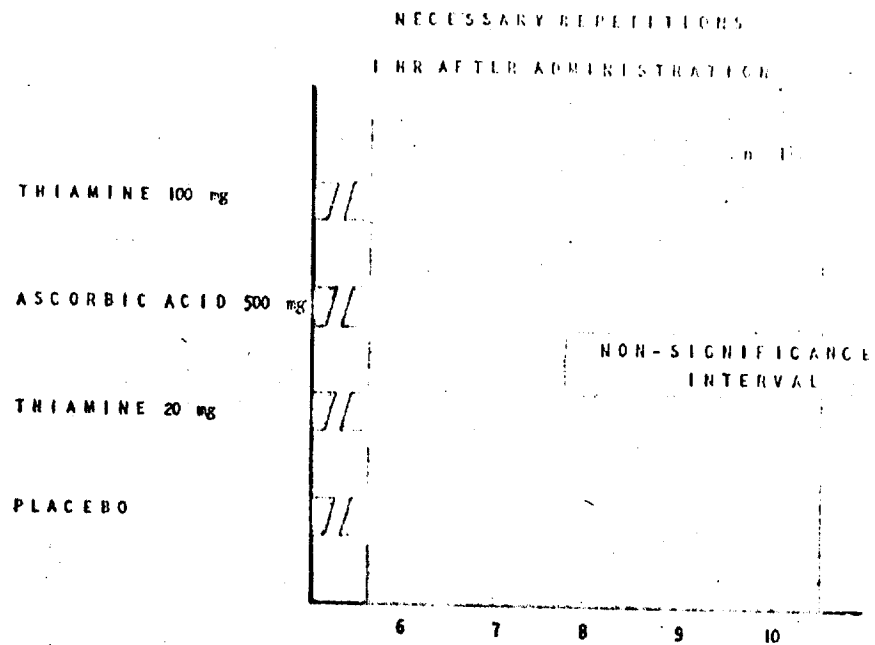


Diagram 1

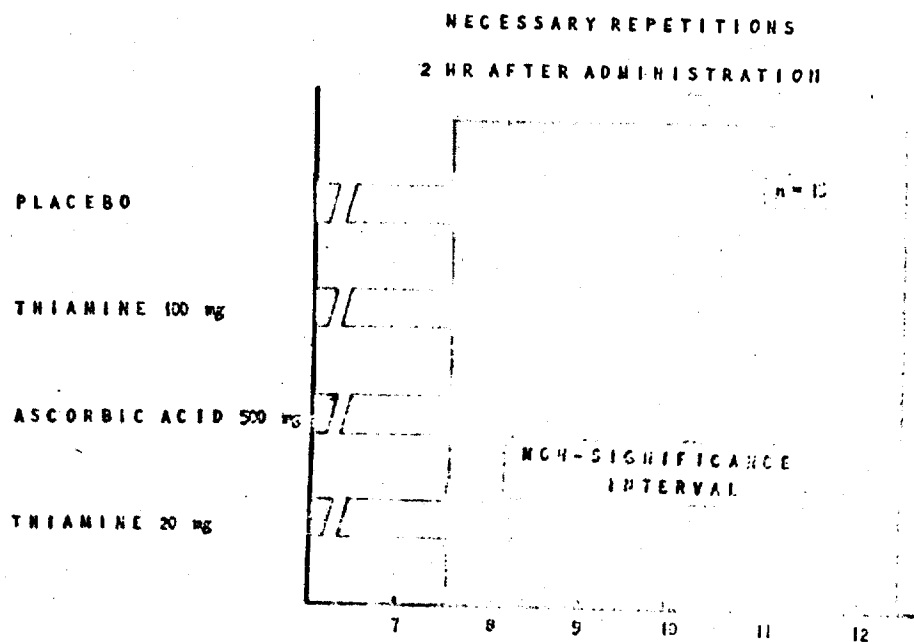


Diagram 2

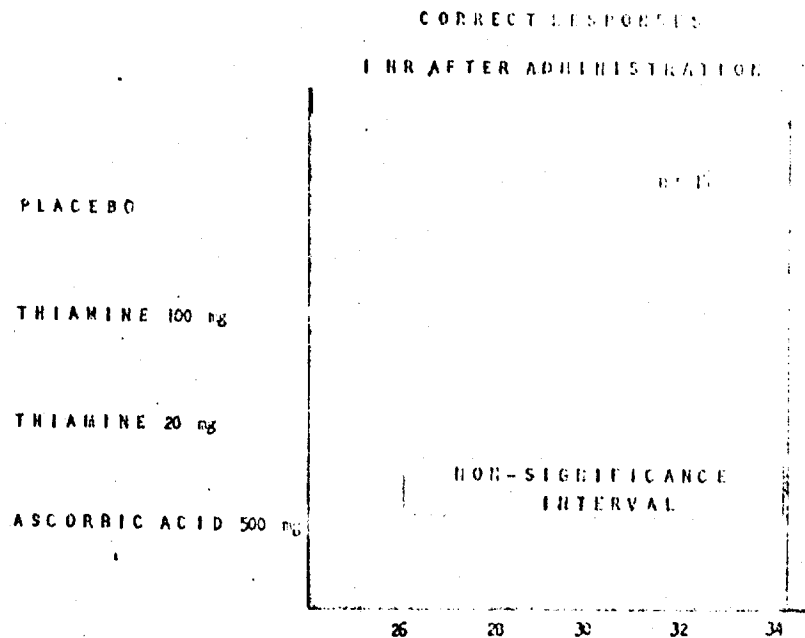


Diagram 3

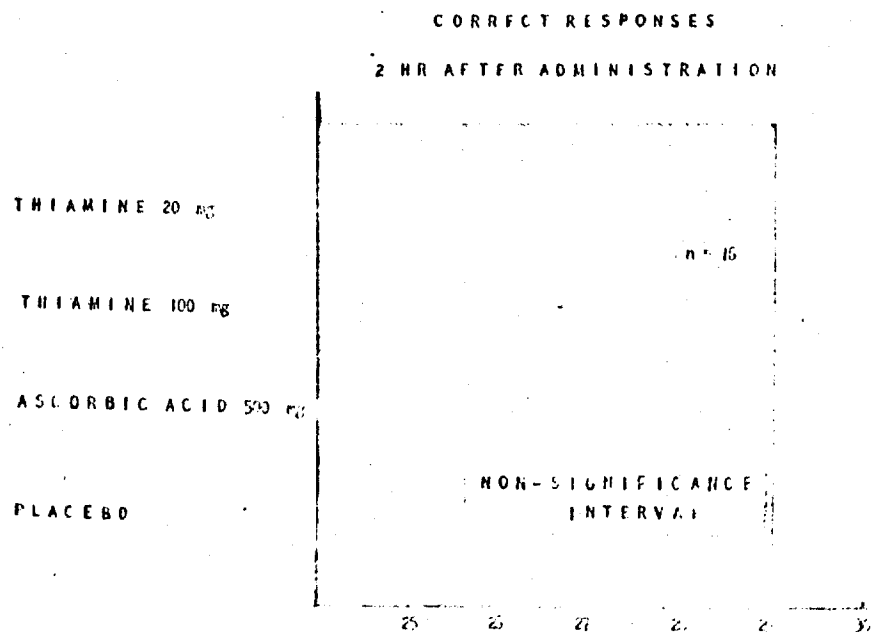


Diagram 4

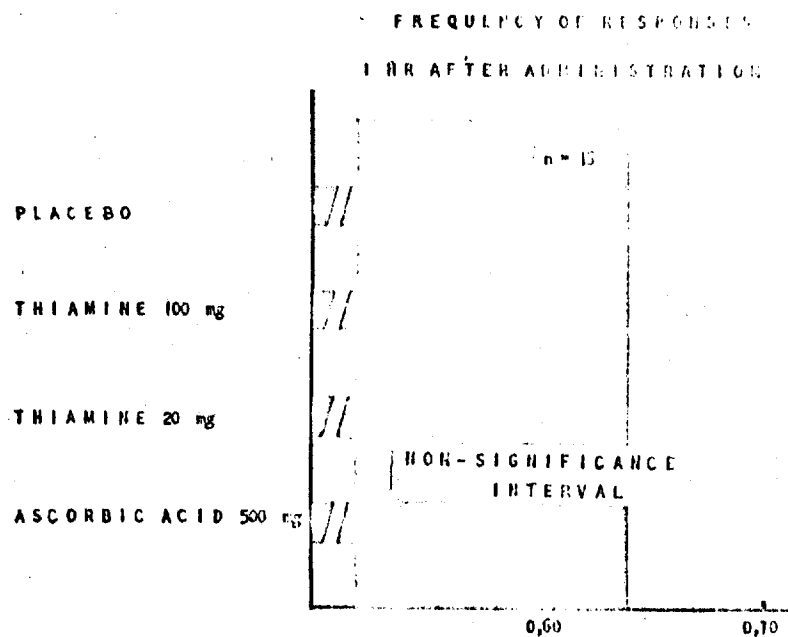


Diagram 5

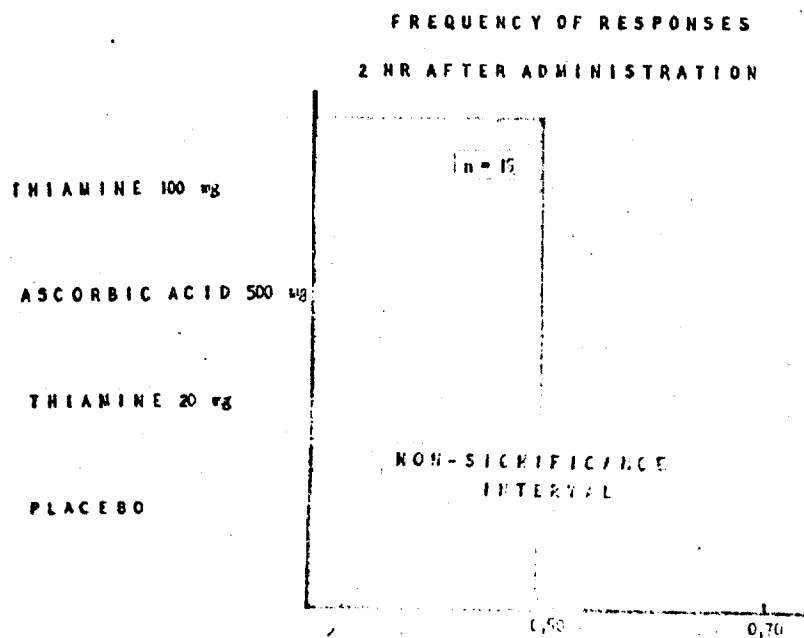


Diagram 6

One hour after the administration of the drugs, differences were apparent in the responses of the persons under investigation ($F = 3.93$ for 15 and 33 d. f.) and in the degree of difficulty to acquire knowledge of the sets of demonstrated objects ($F = 3.72$ for 3 and 33 d. f.). The effect of the levels of the other factors did not reach statistical significance. A significant impairment was, however, observed in the number of correct responses particularly after administration of vitamin C (500 mg). Poorer results were also achieved after application of each of the two doses of vitamin B₁ (20 mg and 100 mg).

Two hours after the administration of the drugs, the responses obtained from the persons under investigation differed significantly ($F = 2.71$ for 15 and 33 d. f.) and pronounced differences also appeared in the degree of difficulty to memorize the sets of laboratory words ($F = 3.18$ for 3 and 33 d. f.). After administration of vitamin B₁ (20 mg and 100 mg), the improvement in the responses was at the level of statistical significance. Very good results were also obtained after administration of vitamin C (500 mg).

Frequencies of responses

In the first experiment, in addition to the different responses of the persons under investigation ($F = 2.19$ for 15 and 33 d. f.), a significant increase in the results obtained from each week in succession ($F = 3.53$ for 3 and 33 d. f.) could be recorded. The effect of the levels of the other factors did not differ significantly.

One hour after the administration of the drugs, similar results were obtained: significant differences in the responses from the subjects ($F = 4.91$ for 15 and 33 d. f.) and an improvement in the responses in the course of the weekly repetitions ($F = 3.97$ for 3 and 33 d. f.). Application of vitamin C (500 mg) resulted in a decrease in the frequency of responses (i. e. prolonged latencies); the values obtained were at the level of statistical significance.

Two hours after the administration of the drugs, significant differences were found in the responses of the persons ($F = 3.04$ for 15 and 33 d. f.).

An analysis of the power-function of the statistical test shows that, with the help of the experiment, the effect of the applied drug can be established with a reliability of 0.95 %, provided that, one hour after administration, the effect of the drug, in comparison with that of the placebo, is by at least 56 % higher in the number of necessary repetitions, by 28 % in the number of correct responses, and by 20 % in the frequencies of responses. From the results of the statistical analysis it is concluded that the effect of the drugs is smaller than the given percentage rates.

Analogously, two hours after administration, the experiment shows the drug as effective if, in comparison with the placebo, its effect is higher by at least 46 % in the number of necessary repetitions, by 29 % in the number of correct responses, and by 23 % in the frequencies of responses. From the statistical analysis it follows again that the effect of the drugs has not reached the given percentage rates.

The means of the effect of the various levels of the factors under control during the experiment are given in Tables 10-14. A comparison between the effect of the drugs under investigation and placebo has been illustrated in Diagrams 1-6.

DISCUSSION

The vitamin B_1 (thiamine, aneurin) is a derivative of pyrimidine and thiazole. It contains both an amino group and bivalent sulphur, which has given rise to its name thiamine. Its presence is absolutely necessary for the normal function of the nervous system.

In the tissues, vitamin B_1 appears bound either to pyrophosphoric acid or to lipoic acid. In combination with pyrophosphoric acid it forms a coferment of carboxylase and dehydrogenase, i. e. the cocarboxylase. A combination of vitamin B_1 with lipoic acid gives rise to lipothiamide which transforms pyruvic acid into acetic acid.

In the organism, vitamin B_1 affects the carbohydrate metabolism. In cases of vitamin B_1 deficiency, lactic acid and pyruvic acid accumulate in the tissues (particularly in the central nervous system). The uptake of dioxide decreases. This may result in seizures or even paralysis. The function of the liver haematopoiesis, and the function of the endocrine glands, etc., are also involved.

Moreover, vitamin B_1 potentiates the effect of acetylcholine. So far, the underlying mechanism is still obscure. It is assumed that it inhibits the effect of choline esterase which hydrolyzes acetylcholine into the less effective choline and acetic acid.

The daily requirement of the human organism is estimated to 1-2 mg of vitamin B_1 . Therapeutically it is administered in doses ranging from 25 to 50 mg daily.

Administration of high single doses of vitamin B_1 is followed by an increase in the metabolic activity and in the responsiveness of the central nervous system. Intravenous infusions of high doses may result in circulatory insufficiency and apnea.²

Zevald¹⁷ studied the effect of vitamin B_1 (10 mg and 20 mg) on the higher nervous activity of normal dogs and on dogs suffering from avitaminosis. Zevald reports that only in dogs with avitaminosis an improvement in the formation of conditioned reflexes is observed whereas in healthy dogs the trend of the effect is to the negative.

Even in man, no stimulating effect of vitamin B_1 on the mental or physical capacity could be demonstrated.^{3, 16}

Graf⁶ observed a statistically significant impairment in the results obtained from persons put to mathematic tests after administration of higher doses of vitamin B_1 . The subjects soon complained of fatigue and hypersomnia.

Michalová¹³ draws attention to the fact that in psychotics, during administration of vitamin B_1 , a greater responsiveness of the nervous system must be taken into consideration. In cases of neurasthenia and exhaustion, the administration of vitamin B_1 may result in an improvement of the general condition. Michalová carried out her experiments on sixty girls (scholarship holders, attendants), who were given daily two doses of vitamin B_1 (50 mg). A stimulating effect on the physical capacity of the persons under investigation could not be demonstrated. On the contrary, the persons complained of drowsiness, fatigability, agitation, etc.

In man, vitamin C (ascorbic acid) has the significance of an actual vitamin. According to Charvát,¹¹ it plays a special role in comparison to the other vitamins. The daily requirement of vitamin C amounts to approximately 50 mg.

which is a multiple of the required quantity of the other vitamins. Clark⁶ assumes that this is attributable to the fact that, in the course of evolution, ascorbic acid has become a vitamin much later than the other vitamins. This finding offers an explanation for the relatively high tolerance of ascorbic acid.

Several findings made by different authors prompted us to study the effect of a single higher dose of ascorbic acid on the higher nervous activity in man. In the first place it was the fact that the brain is one of the organs having the highest level of ascorbic acid. In the brain, its presence is absolutely necessary for the metabolism of phenylalanine, tyrosine, and phenylpyruvic acid. Hess⁷ pointed out that ascorbic acid had an inhibitory effect on adenosine triphosphatase which was activated by Mg^{++} , Na^+ , and K^+ ions. Glynn^{4,5} found that ascorbic acid produced an inhibitory effect on the transfer of sodium and potassium through the cell membrane.

The adenosine triphosphatase activity is a necessary requirement for the development and maintenance of the concentration level of potassium and sodium ions between the extracellular and the intracellular medium. A disorder in the concentration of these ions is followed by a decrease in the potential of the cells of the central nervous system. The neurons tend to depolarization and to discharges.

Adenosine triphosphatase contains SS-groups in its prosthetic component. Their reduction results in the reversible inhibition of the activity of the enzyme. It is assumed that ascorbic acid may reduce these groups either directly or indirectly with the help of glutathione.

Sklenovský¹⁴ reports that the common feature of inhibitors which interfere with the transfer of sodium, potassium, and block the adenosine triphosphatase as for example ascorbic acid, ouabain and EDTA - is the liberation of glutamic acid from the intracellular medium and its transfer into the extracellular medium. Sklenovský studied this phenomenon in vitro. He suggests that the same process might take place in vivo, particularly after application of high doses of ascorbic acid. To his opinion, higher doses of ascorbic acid have a stimulating effect on the central nervous system.

The administration of large doses of ascorbic acid may sometimes result in the development of vagotonia in children or, in adults, in increased diuresis up to dehydration of the organism.¹⁵

Higher concentrations of ascorbic acid may also result in a higher level of reduced glutathione in the tissues, the secondary effect of which would be a modification of the activity of various enzymic systems and thus of the whole metabolism. It is generally known that by affecting the SS- and the SH-groups, glutathione has an activating or inhibitory effect on approximately 18 enzymes.¹

Jánský¹² is of the opinion that in the tissue, ascorbic acid promotes hydroxylation of phenylalanine and tyrosine to catecholamines, it accelerates the hydroxylation of desoxysteroids to steroids, it facilitates the transfer of electrons, and it brings about oxidation without phosphorylation.

Our experimental series was designed to study, during the first two hours after administration of vitamin B₁ (20 mg and 100 mg) and of vitamin C (500 mg), the effect of these two vitamins on the formation, fixation, and the latencies of artificial conditioned speech connections in healthy subjects.

In experiments carried out one hour after application of vitamin B₁ (100 mg) and vitamin C, a more pronounced, but statistically insignificant impairment was

observed in the number of necessary repetitions. A very marked impairment (the values were at the level of statistical significance) was encountered in the number of correct responses and, particularly, in the frequencies of responses after a single high dose of vitamin C (500 mg).

Two hours after the administration of the vitamins, each of the two doses of vitamin B₁ brought about a more pronounced improvement in the number of correct responses. The number of necessary repetitions was lowest after application of a 20 mg dose of vitamin B₁. The frequencies of the responses differed only slightly.

When the method of artificial conditioned speech connections is made use of, the results obtained indicate that, one hour after administration, ascorbic acid (500 mg) produces a prolongation of the latencies of the responses and a decrease in the number of correct responses. Each of the two doses of vitamin B₁ has a favourable effect however, only two hours after application of the vitamin, i. e. they improve the fixation of artificial conditioned speech connections (the number of correct responses shows to be increased).

SUMMARY

(1) The method of artificial conditioned speech connections was used to study the acute effect of single, higher, perorally administered doses of vitamin B₁ (20 mg and 100 mg thiamine), vitamin C (500 mg), and placebo on the higher nervous activity of 16 healthy and unfatigued volunteers - university students (8 females and 8 males, age ranging from 17-18 years). The experiments were carried out during the morning and forenoon hours.

(2) The investigation of the subjects was always performed before, and one and two hours after the administration of the drugs. The sources of variability were designed on the hyper-graceo-latin square. The criteria for the assessment of the results were the number of necessary repetitions, then the number of correct responses, and the frequencies of responses during the first eight repetitions (testing of the active knowledge of the subjects).

(3) In experiments carried out one hour after the administration of the drugs, a statistically insignificant impairment was observed in the number of necessary repetitions after administration of vitamin B₁ (100 mg) and vitamin C (500 mg), and a pronounced impairment (the values were at the level of statistical significance) in the number of correct responses, and in the frequencies of responses after application of vitamin C (500 mg).

Consequently, application of a single dose (500 mg) of ascorbic acid results in a significant prolongation of the latencies of the responses and in a decrease in the number of correct responses when the artificial conditioned speech connections are elaborated.

(4) In experiments carried out two hours after administration of each of the two doses of vitamin B₁, a marked improvement is observed in the number of correct responses (the values are close to the level of statistical significance).

(5) On the basis of an analysis of the power-function of the statistical test, the sensitivity of the method made use of has been established. The method of artificial conditioned speech connections enables us, under the given experimental conditions and the same extent of the experiment, to determine (with a reliability

вильных ответов и в. 20 % для фреквенции ответов, а для действия не меньше чем на 46 % для числа правильных ответов и 23 % для фреквенции ответов.

SLEDOVÁNÍ AKUTNÍHO VLIVU NĚKTERÝCH LÁTEK NA VÝŠI NERVOVOU ČINNOST ČLOVĚKA. ČASŤ VIII. THIAMIN (20 mg a 100 mg), KYSELINA ASKORBOVÁ (500 mg).

Souhrn

1. Autoři sledovali metodu umělých podmíněných řečových spoju vliv jedno-
rázových, vyšších, perorálně podaných dávek vitamínu B₁ (20 mg a 100 mg
thiaminu), vitamínu C (0,5 g kyseliny askorbové) a placebo na vyšší nervovou
činnost 16 zdravých a neuvážených dobrovolníků vysokého věku (6 žen, 8 mužů,
věkové rozpětí 18-19 let) v dopoledních hodinách.

2. Vyšetřování osob bylo provedeno vždy před podáním, za hodinu a za dvě
hodiny po podání látek. Zdroje variability byly rozplňovány na hyper-řečko-
latinský čtverec. Kriteřiem hodnotení výsledků byl počet potřebných opakování,
a dále počet správných odpovědí a frekvence odpovědí u prvních osmi opaková-
ních (zkoušení aktivní znalosti vyšetřovaných osob).

3. V pokusech za hodinu po podání látek zaznamenali autoři statisticky ne-
významné zhoršení v počtu potřebných opakování po aplikaci 100 mg vitamínu
B₁ a 0,5 g vitamínu C a nápadně výrazné zhoršení (hodnota je na hranici sta-
tistické významnosti) v počtu správných odpovědí a frekvence odpovědí po apli-
kaci 0,5 g vitamínu C.

Kyselina askorbová v jednorázové dávce 0,5 g tedy výrazně prodlužuje latenci
odpovědi a snižuje počet správných odpovědí při vypracování umělých podmí-
něných řečových spoju.

4. V pokusech za dvě hodiny bylo pozorováno výraznější zlepšení v počtu
správných odpovědí po aplikaci obou dávek vitamínu B₁ (hodnoty se blíží sta-
tistické významnosti).

5. Rozborem silofunkce statistického testu určili dále autoři citlivost použitáho
metodického přístupu k hodnotení vlivu studovaných látek na vyšší nervovou
činnost člověka. Metoda umělých podmíněných řečových spoju za daných exper-
imentálních podmínek a při stejném rozsahu experimentu spolehlivě (s 95 %
spolehlivostí) odhalí vliv studované látky, jestliže se po jedné hodině po pře-
bání farmakologického změny měření vzhledy - ve cross-over testu - dávkou placebo
placebo - klesne počet potřebných opakování o 25 % a frekvence odpovědí o 25 %
užších odpovědí a o 25 % ve frekvenci odpovědí. Jestliže se po jedné hodině po pře-
bání placebo - klesne počet potřebných opakování o 25 % a frekvence odpovědí o 25 %
užších odpovědí a o 25 % ve frekvenci odpovědí.

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Translated by Jan Hrbek

Kato, H.: THE EFFECT OF B VITAMINS ON THE BIOSYNTHESIS OF PROTEIN-BOUND LIPOIC ACID. III. INFLUENCE OF THE OVERDOSE OF B VITAMINS ON THE BIOSYNTHESIS OF PROTEIN-BOUND LIPOIC ACID. Bitamin, Vol. 31 (4), pp. 309-314, 1965. Department of Pediatrics, Kyoto Prefectural University of Medicine, Kyoto.

With increasing use of vitamin preparations, particularly active vitamin preparations, the problems due to massive vitamin intake has recently drawn attention. One of the problems that require attention is their effect on the metabolism of other vitamins. In 1940, Sydenstricker et al. (1) observed the aggravation of B₁ and B₂ deficiency syndrome in pellagrins given massive doses of nicotinic acid, and Leitner et al. (2) also called attention to a similar phenomenon. In Japan, Inoue et al. (3) continuously administered large doses of TPD (thiamine propyldisulfide), a derivative of vitamin B₁, and noted vitamin B₂ deficiency syndrome in the patients, but Miyagawa et al. (4,5) failed to observe any apparent change in the organs or any disorder due to large dose administration of B₁. In the previous 2 papers, the author discussed the effect of B₁, B₂, and B₆ deficiencies on the biosynthesis of protein bound LiA (lipoic acid). In the present experiment, he studied whether or not massive administration of B₁, B₂, and B₆ could influence the LiA metabolism, particularly the biosynthesis of bound LiA.

EXPERIMENTAL PROCEDURE

(1) EXPERIMENTAL ANIMALS AND FEED

Forty days old male Wistar white rats weighing about 50 g were raised on the standard solid feed for 4 - 6 days, and divided into 9 groups. The feeds given to these groups contained B₁-HCl at 1 mg (group II), 10 mg (group III), and 50 mg (group IV) per kg of body weight, TTFD (thiamine tetrahydrofurfuryldisulfide) at 1, 10, and 50 mg per kg b.w. (V, VI, and VII groups, respectively), B₂ at 1 mg per kg of b.w. (group VIII), and B₆ at 1 mg per kg of b.w. (group IX). After the animals were raised on the feeds for a specific period of time, they were killed by decapitation, and examined. The amount of B₁, B₂, and B₆ contained in the standard solid feed was 0.2 (mg%). The amount of feed, given upon demand, was 10 - 20 g per animal daily.

(2) DETERMINATION OF THE BIOSYNTHETIC LEVEL OF PROTEIN BOUND LiA.

As in the previous experiment, Nose's method was employed (6). The nitrogen level of liver Mit (mitochondria) was measured by micro-Kjeldahl method.

(3) DETERMINATION OF LIVER GOT ACTIVITY

Reitman-Frankel's method was employed (7). Ground liver solutions diluted 1000 and 500 fold were prepared with 0.1 M phosphate buffer at pH 7.4, and permitted to react at 37°C for 60 minutes. The activity was examined in terms of the amount of the resultant oxaloacetic acid.

EXPERIMENTAL RESULTS

(1) GROWTH AND BODY WEIGHT

As shown in Figure 1, the control group (group I) indicated a linear growth curve, and the massive B₂ group (VIII), a similar pattern of growth. The growth of the massive B₁-HCl groups (II, III, and IV) was more favorable than the control group for the first 2 weeks, and the TTFD groups, V and VI, also showed a favorable growth pattern, their results being close to that of the B₁-HCl groups. However, group VII which received an extremely large dose of TTFD, at 50 mg/kg, showed suppressed growth. The massive B₆ group (IX) exhibited a pattern different from the B₁ massive dose groups, with a temporary drop in weight gain. It was noted, however, that groups I, II, VIII, and IX which remained under observation for longer periods exhibited gradual diminution of the effect on body weight, with their body weight returning to the control level in 4 weeks.

(2) BIOSYNTHESIS OF PROTEIN BOUND LiA.

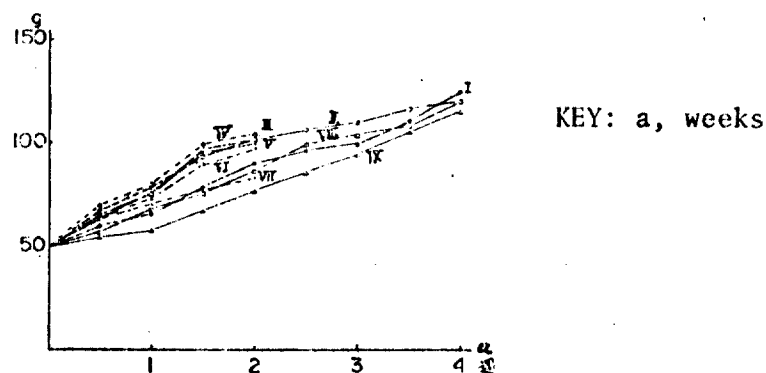


Figure 1. Growth Curves

TABLE 1. BIOSYNTHETIC ACTIVITY OF
PROTEIN BOUND LiA (cpm/mg N)

投与量(mg/kg)	飼育期間(週)	1	2	3	5
c. 対照		3914±175*	3825±245	4012±213	3985±303
B ₁ -HCl	1	5137±109	5362±213	4308±381	4125±363
B ₁ -HCl	10	—	5300±445	—	—
B ₁ -HCl	50	—	5423±303	—	—
TTFD	1	—	5575±496	—	—
TTFD	10	—	5300±314	—	—
TTFD	50	—	3625±511	—	—
B ₂	1	4057±237	3874±219	4123±251	4087±306
B ₆	1	3908±285	2687±212	3578±262	3811±251

d * 標準偏差

KEY: a, dose (mg/kg); b, duration (weeks); c, control; d, standard deviation

As of 2 weeks from the onset of experiment, the massive B_1 -HCl groups (II, III, and IV) and the TTFD massive dose groups (V, VI) exhibited the highest protein bound LiA forming capacity of the liver MiT, but the B_2 massive dose group (VIII) showed no difference from the control as shown in Table 1 and Figures 2 and 3. The values of the TTFD-50 mg/kg group (VII) were slightly lower than the control level. The biosynthesis of LiA in the B_6 group (IX) was clearly suppressed. In the 4th week, however, the groups given 1 mg/kg of B_1 , B_2 , and B_6 (groups II, VIII, and IX) showed no difference.

(3) LIVER GOT ACTIVITY

The determination of liver GOT activity revealed no apparent difference between the groups.

(4) CHANGES IN THE BOUND LiA OF LIVER MiT IN RELATION TO TIME FOLLOWING THE ADMINISTRATION OF LiA- ^{35}S .

LiA- ^{35}S was intraperitoneally administered to groups II and IX at 20 mg/kg in the 2nd week after the onset of administration, and the incorporation of the substance into the liver MiT protein was studied in relation to time. The results are illustrated in Table 3 and Figure 4. As noted in the graph, the B_1 groups gave the same results as the control as of 30 minutes later, but the B_6 group showed a value 70% lower than the control. In either case, the value dropped rapidly, with no difference among the groups in 6 hours.

(5) EFFECT OF OTHER COMPOUNDS

B_1 , CoC (cocarboxylase), B_6 , and PAL-P (pyridoxal phosphate) were added to liver MiT of the control group in vitro, and their effect on the biosynthesis was studied. As shown in Table 4, the compounds produced no significant effect.

(6) NITROGEN LEVEL IN LIVER MiT

As shown in Figure 5, no significant difference between the groups was noted.

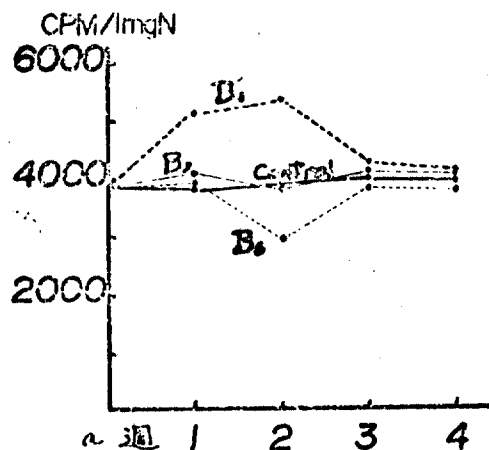


Figure 2. Synthetic Activity of Protein-Bound LiA.
(KEY: a, weeks)

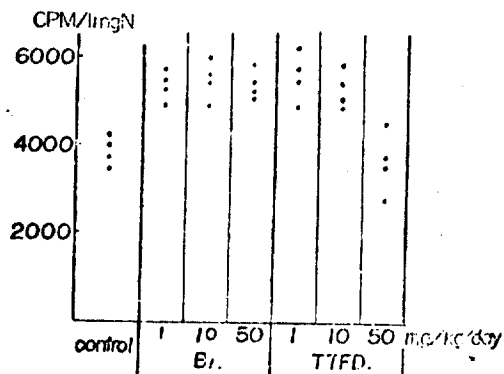


Figure 3. Synthetic Activity of Protein-Bound LiA.

TABLE 2. LIVER GOT ACTIVITIES IN THE WHITE RATS GIVEN LARGE DOSES OF VITAMINS (pyruvic acid u mol/mg/hr)

a 投与量 (mg/kg)		b 飼育期間 (週)		1	2	3	4
c 対 照				1.21	1.13	1.24	1.18
Bi-HCl	1			1.17	1.19	1.11	1.22
Bi-HCl	10			—	1.25	—	—
Bi-HCl	50			—	1.04	—	—
TTFD	1			—	1.18	—	—
TTFD	10			—	1.21	—	—
TTFD	50			—	1.06	—	—
B ₂	14			1.18	1.23	1.22	1.10
B ₆	1			1.23	1.14	1.17	1.14

KEY: a, dose (mg/kg); b, duration (week)

TABLE 3. INCORPORATION OF LiA-³⁵S INTO LIVER MiT PROTEIN (in vivo)

投与後時間 (分)	対 照	B ₁ 大量 (II)	B ₆ 大量 (IV)	投与後時間 (分)	対 照	B ₁ 大量 (II)	B ₆ 大量 (IV)
30	3156	2992	2240	120	681	870	448
	2550	2430	1728		516	628	260
f 平 均	2853	2711	1984	f 平 均	600	749	354
60	2128	2171	1251	360	306	471	157
	1582	1737	1053		124	231	99
f 平 均	1855	1954	1102	f 平 均	215	351	123

g 単位 = cpm/mg N

KEY: a, time (min) after administration; b, control; c, B₁-large dose (II); d, B₆-large dose (IV); e, time (min) after administration; f, average; g, unit of measurement

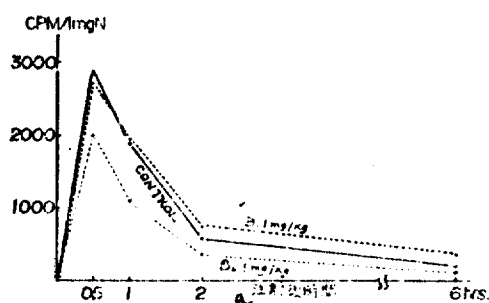


Figure 4. Uptake of LiA-³⁵S into Liver MiT Protein. (in vivo)
(KEY: a, time after injection)

TABLE 4. EFFECTS OF B₁, CoC, B₆, and PAL-P ON THE BIOSYNTHESIS OF PROTEIN BOUND LiA.

a 添 加	cpm/mg N
—	3950
B ₁	3797
CoC	3885
B ₆	3416
PAL-P	4070

B₁, CoC 2 × 10⁻⁵M B₆, PAL-P 4 × 10⁻⁵M

KEY: a, Agent added.

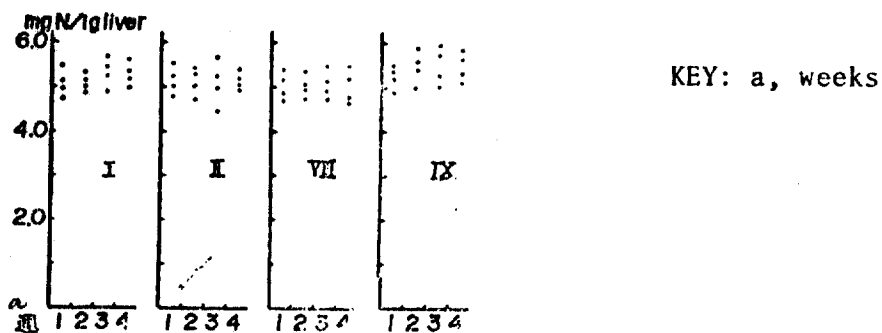


Figure 5. Nitrogen Levels in Liver Mit.

DISCUSSION

With the recent progress in vitaminology, particularly in conjunction with the advances in the research on the coenzyme action and physiologic activity of vitamin B compounds, various preparations, such as those called "active", "coenzymic", or "internally retainable", have been produced and administered at massive doses in various clinical fields, primarily for their pharmacologic action rather than their inherent actions as vitamins. The research efforts and achievements centering around B₁ in the biochemical, pharmacologic, and clinologic disciplines, in Japan have been remarkable, and in our pediatric field, the use of B₁ and its derivatives at massive doses has been expanded to such areas as neurologic, muscular, and nutritional disturbances, rheumatic disorder, and neurotonia. On the other hand, however, some investigators assert that the massive administration of B₁ increases the excretion of other vitamin B compounds or disrupts their phosphorylation. Thus, it is deemed imperative to thoroughly investigate the degree and mechanism of disturbance in the utilization of other vitamins and the biochemical side effects due to massive intake of a specific form of vitamin.

The authors conducted the above experiment, and obtained a series of data. When B₁-HCl was orally administered to young white rats at 1, 10, and 50 mg per kg of body weight daily for 4 weeks, their weight gain during the first 1 - 2 weeks surpassed that of the control which had been given B₁ at a daily dose of 20 - 40 u, with clear signs of stimulated biosynthesis of protein bound LiA in the liver Mit. However, in 3 - 4 weeks, both the body weight and the biosynthesis of bound LiA were reduced to the control level despite the continued massive B₁ administration. When TTFD was given at large doses, i.e., at the molar equivalent to the dose of B₁-HCl, both factors indicated a rise as in the administration of B₁-HCl, at doses corresponding to 1 and 10 mg/kg of B₁-HCl, but at the dose corresponding to 50 mg/kg, the weight gain dropped below the control level in 1 - 2 weeks, and the bound LiA synthesizing activity was no more favorable than that of the control. On the other hand, the administration of B₂ at 1 mg per kg of body weight daily produced no change in the growth curve, or the biosynthesis of bound LiA, while the same amount of B₆ caused the weight gain to drop

below the control level, with suppressed biosynthesis of bound LiA in 1 to 3 weeks. However, these effects of B₆ diminished in the 4th week despite the continued administration of the agent, with the weight gain and LiA biosynthesis being at the same levels as those of the control.

Summarizing the experimental results, B₂ exerted no significant effect, but the massive dose of B₁ or B₆ produced changes in body weight and the synthetic activity of LiA in a parallel manner. These changes, however, subsequently diminished despite continued intake of B₁ or B₆. These results can be interpreted as that, following the massive administration of B₁ or B₆, the growth and metabolism of a living organism is subjected to a wide range of effect including an influence on weight gain, and the stimulatory or inhibitory effect on the biosynthesis of bound LiA is secondary to the change in the growth rate and metabolism. It is also postulated that, while a living organism continues to receive massive dose of the vitamin, it readjusts its metabolic activity, or manifests its ability to eliminate the changes by producing a B₁ or B₆ combating mechanism or an adaptable enzyme system. The change occurring in the biosynthesis of bound LiA is not a direct effect of B₁, CoC, B₆, or PAL-P as is evidenced by the fact that the direct addition of these compounds to the liver Mit of the control animals produced no specific change.

With regard to the nature of the metabolic change produced by massive B₁ administration, no specific discussion can be made at this point due to lack of detailed investigation. As stated in the beginning in relation to Klopp's report (8), massive dose of B₁ results in an increased excretion of B₂ several hours later. Inoue (3) also observed that TPD-treated patients developed vitamin B₂ deficiency symptoms. According to Shinagawa (9), large dose administration of B₁ and TAD (thiamine allyldisulfide) was followed by an increased urinary excretion of nicotinic acid and B₂. Several years earlier, Cherillard et al. (10) stated that the phosphorylation reaction of B₁ is disrupted by B₂ and B₆, asserting that a molar equivalent of B₆ completely disrupts the esterification of B₁ while in the presence of a large amount of B₁, the phosphorylation of B₆ is disturbed. Moreover, Richards (11) reported that young white rats developed vitamin B₆ deficiency symptoms upon massive administration of vitamin B₁, and other vitamin deficiencies subsequently. These experimental observations suggest the complex competitive phenomena among B₁, B₂, and B₆ in relation to their phosphorylation reaction. In Murata's experiment in which B₁ was given to laboratory animals at various doses, no change in organ B₂ level due to the B₁ was noted. Although these studies are the major research efforts concerning the metabolism of massive B₁ intake, the results do not seem to provide any direct clue to the interpretation of our experimental results.

Mibu of this faculty (12) previously held that the composition of amino acids or proteins in the diet exerts a significant effect on the biosynthesis of bound LiA. Thence, we assumed that the massive dose of B₁ may influence the utilization of the proteins in the diet, and the biosynthesis of bound LiA, subsequently. However, the nitrogen level in the liver Mit in this experiment indicated no appreciable difference among the groups, failing to provide any substantial ground for

the above assumption. When the dose was increased to 50 mg/kg, the effect of B₁ differed from that of TTFD. There is no simple explanation to this phenomenon. TTFD can be absorbed through the intestinal tube more readily than B₁-HCl. Therefore, its concentration gradient at the time of internal uptake should naturally be higher than that of B₁-HCl. Thus, it is yet to be investigated whether the successive accumulation of such differences resulted in the kind of outcome the author obtained from the present investigation, whether there is an optimum range of dose for B₁ with regard to its effect on the body weight and biosynthesis of bound LiA, or whether the structural difference between B₁ and TTFD produced the difference in pharmacologic effect as a result of the massive dose.

The adverse effect of massive B₆ administration on the growth and biosynthesis of bound LiA has already been discussed. Which of the metabolic systems is subject to the primary change is yet to be clarified. Yamada (13) intravenously injected B₁, B₆, and their derivatives at 50 mg, and observed the depression of the blood sugar level and the elevation of the blood insulin activity level by TTFD in 2 hours, and the elevation of the blood insulin activity level by B₆ and PAL-P. He also found that intraperitoneal injections of TTFD, B₆, and PAL-P to hypophysectomized and adrenalectomized white rats caused a drop in blood sugar. Moreover, he administered B₆ and TTFD to humans by the intravenous route at 100 mg, and observed an increased excretion of 17-OHCS into the urine 1 hour later. These results suggest that massive administration of B₆ influences various endocrine systems such as the adrenals and pancreas, and these general effects produced secondary changes such as a change in body weight and activation of LiA, but a closer study is yet to be made.

LiA-³⁵S was injected to white rats which had received massive doses of B₆ for the preceding 2 weeks, and the formation of bound LiA in vivo was investigated. As illustrated in Table 3 and Figure 4, the values were significantly lower than the control level. As has been maintained by Cherillard et al., the internal phosphorylation of B₆ may be competing with the reaction depending upon ATP which is also required for the biosynthesis of bound LiA. Examining the results of this study, it is found that such an effect is temporary, and the prolongation of the administration of B₆ is of no significance.

In a series of 3 papers, the author has discussed the correlation between various water soluble vitamins and the formation and maintenance of bound LiA. The results of the experiment varied according to the type and dose of the vitamins and were highly characteristic. Some of the results are yet to be analyzed and explained, but since these experimental facts are immediately related to our clinical practice, they should be used as the foundation for the elucidation of their action mechanism and proper clinical application.

CONCLUSION

B₁-HCl, TTFD, B₂ and B₆ were orally administered to young white rats at massive doses and the following results were obtained.

1. The administration of B₁-HCl stimulated the biosynthesis of protein bound LiA regardless of its dose.

2. TTFD at 1 and 10 mg/kg per body weight produced the same effect as B₁-HCl, but at 50 mg/kg, the biosynthesis of bound LiA was reduced.

Massive dose of B₂ exerted no effect on the biosynthesis of protein bound LiA.

4. Massive administration of B₆ temporarily depressed the biosynthetic activity of protein bound LiA.

The summary of this paper was presented at the 16th meeting of the Society of Vitaminology, and the 153rd meeting of the Committee for the Study of Vitamin B.

The author acknowledges the valuable assistance extended by Prof. Nakamura and Dr. Kusunoki.

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蛋白結合型リポ酸の生成におよぼすB群ビタミンの影響

(Ⅲ) ビタミンB₁, B₂, B₆大量投与時における蛋白結合型リポ酸の生成

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加 藤 英 彦

THE EFFECT OF B VITAMINS ON THE BIOSYNTHESIS OF PROTEIN-BOUND LIPOIC ACID

(III) INFLUENCE OF THE OVER-DOSE OF B VITAMINS ON THE BIOSYNTHESIS OF PROTEIN-BOUND LIPOIC ACID

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Influences of daily administration of a large amount of thiamine, thiamine tetrahydrofurfuryl disulfide (TTFD), riboflavin and pyridoxine to rats for a month were investigated by observations on the growth of rats, the biosynthesis of protein-bound lipolic acid in liver mitochondria and the activity of glutamic oxalacetic transaminase in liver homogenate. Over-dose of thiamine (1,10 and 50mg/kg) and TTFD (1 and 10mg/kg) induced an increased biosynthetic activity of protein-bound lipolic acid, but over-dose of TTFD (50mg/kg) and pyridoxine induced a decreased biosynthetic activity. Over-dose of riboflavin did not effect the biosynthetic activity of protein-bound lipolic acid. The activity of glutamic oxalacetic transaminase was not affected in those states.

近年ビタミン剤、とくにいわゆる活性型ビタミン剤の普及につれてビタミンの大量投与にかんする問題がとりあげられるようになったが、このばあいには他種のビタミン代謝にたいしてなんらかの影響をおよぼす可能性は当然注目されることである。すなわち、すでに1940年にSydenstrickerら¹⁾はペラグラの患者にニコチン酸の大量投与を行なつたさい、B₁, B₂欠乏症候がかえつて増悪することを認めたのをはじめとして、Leitnerら²⁾も同様のことを指摘している。わが国においても井上ら³⁾はB₁の誘導体であるTPD^{*)}の大量を患者に連用したばあいにB₂欠乏症候のおこることを認めているが、宮川ら⁴⁾はB₁大量投与により肝臓内のB₂量に著変はみられず大量投与による障害はなかつたと報告し一定した結論は得られていない。前2報で著者はB₁, B₂, B₆の欠乏が蛋白結合型LiA^{*)}の合成能におよぼす影響について報告したが、今回はB₁, B₂, B₆の大量投与によりLiA代謝ことに結合型LiAの合成能が影響を受けるものであるか否かについて検討した

のでその結果を報告する。

実 験 方 法

(1) 実験動物および飼料

実験動物としては生後10日目、体重30g前後のWistar系雄シロネズミを用いた。まず標準固型飼料で1〜6日間飼育したのも9群にわけそれぞれ標準固型飼料に体重1kgあたりB₁-HClを1mg(II群)、10mg(III群)、50mg(IV群)、TTFD^{*)}を1mg(V群)、10mg(VI群)、50mg(VII群)、B₂を1mg(VIII群)、B₆を1mg(IX群)を毎日添加し一定期間飼育後に頸動出血死せしめ実験に供した。なお標準固型飼料の含量(mg%)はB₁0.2, B₂0.2, B₆0.2であつた。飼料は1日1匹あたり10〜20gを自由に摂食させた。

(2) 蛋白結合型LiAの合成能の測定

前報と同様に飯島⁵⁾の方法に従つて測定した。なお肝Mit^{*)}窒素量はMicro-Kjeldahl法によつて測定した。

^{*)} TPD=Thiamine propyldisulfide ^{*)} LiA=Lipoic acid

^{*)} TTFD=Thiamine tetrahydrofurfuryldisulfide ^{*)} Mit=Mitochondria

(3) 肝 GOT 活性の測定

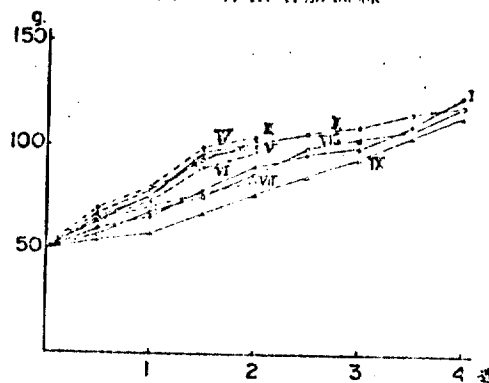
Reitman-Frankel 法⁷⁾ に準じて施行した。すなわち 0.1M, pH7.4 のリン酸緩衝液で肝の1000および500倍懸濁液をつくり37°C, 50分間反応させたのち生成したオキサロ酢酸の量をもってその活性の指標とした。

実 験 成 績

(1) 発育状態および体重

図1に示すように対照群(I群)では体重はほぼ直線的に増加し、Br大量投与群(Ⅷ)もほぼこれと平行していた。一方、Br-HCl大量投与群(Ⅱ, Ⅲ, Ⅳ)では飼育2週目まではI群より発育は良好で、TTFDを投与したV, VI群も発育がよくBr-HCl投与群とはほぼ同等の結果が得られた。しかし50mg/kgというさきわめて大量に投与したⅦ群では発育はかえって抑制される傾向が認められた。Br大量投与群(Ⅸ)ではBr大量投与群とは異なつた態度をしめし、体重増加は一時的に抑制され

図1 体重増加曲線



た。しかし大量投与の体重におよぼす影響はさらに投与を続け観察した群(I, Ⅱ, Ⅷ, Ⅸ)ではしだいに軽度となり、4週目にはそれぞれ対照と同等の体重になった。

(2) 蛋白結合型 LiA の生成

表1 蛋白結合型 LiA 合成能 (cpm/mg N)

投与量 (mg/kg)	飼育期間 (週)	1	2	3	5
対 照		3914 ± 175*	3825 ± 245	4012 ± 213	3985 ± 303
Br-HCl	1	5137 ± 109	5362 ± 213	4308 ± 381	4125 ± 363
Br-HCl	10	—	5500 ± 445	—	—
Br-HCl	50	—	5423 ± 303	—	—
TTFD	1	—	5575 ± 496	—	—
TTFD	10	—	5300 ± 314	—	—
TTFD	50	—	3625 ± 514	—	—
Br	1	4057 ± 237	3874 ± 219	4123 ± 251	4087 ± 306
Br	1	3608 ± 285	2687 ± 212	3578 ± 262	3811 ± 251

* 標準偏差

図2 蛋白結合型 LiA 合成能

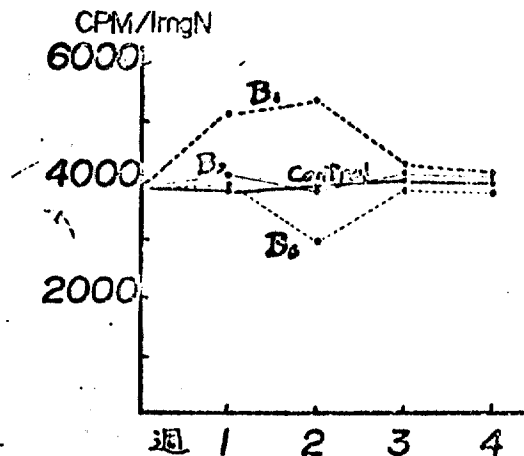
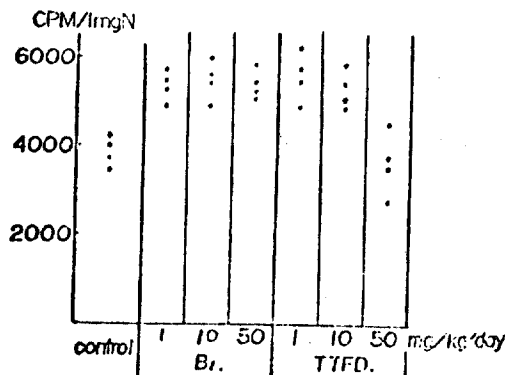


図3 蛋白結合型 LiA の合成能



飼育2週後の肝 M₁₀ における蛋白結合型 LiA の合成能をみると表1, 図2, 3に示すように Br-HCl 大量

投与群(II, III, IV)およびTTFD大量投与群(V, VI)がもつとも高く、B₆大量投与群(VII)は対照と差がなかった。TTFD 50mg/kg 投与群(VII)では対照よりやや低い結果を得た。B₆投与群(IX)では合成能は著明に低下していた。しかしながらB₁, B₂, B₆を1mg/kg投与した群(II, VII, IX)について、1, 2, 3, 4週と経過を追つてみると、いずれも4週後にはほとんど差が認められなくなった。

(3) 肝 GOT 活性

各群について肝 GOT 活性を測定したが表2にみる

表2 ビタミン大量投与シロネズミの肝 GOT 活性(ピルビン酸 μ モル/15分)

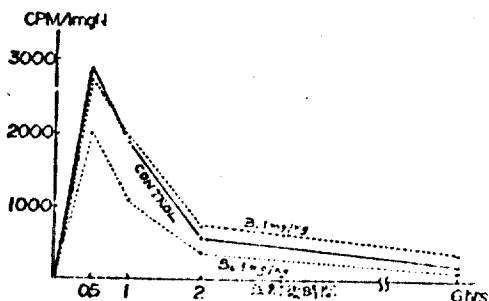
投与量(mg/kg)	飼育期間(週)	1	2	3	4
対 照		1.21	1.13	1.24	1.18
B ₁ -HCl	1	1.17	1.19	1.11	1.22
B ₁ -HCl	10	—	1.25	—	—
B ₁ -HCl	50	—	1.01	—	—
TTFD	1	—	1.18	—	—
TTFD	10	—	1.21	—	—
TTFD	50	—	1.06	—	—
B ₂	1	1.18	1.23	1.22	1.10
B ₆	1	1.23	1.14	1.17	1.14

表3 LiA-³⁵Sの肝Mit 蛋白へのとりこみ (in vivo)

投与後時間(分)	対 照	B ₁ 大量(II)	B ₆ 大量(IV)	投与後時間(分)	対 照	B ₁ 大量(II)	B ₆ 大量(IV)
30	3156 2550	2992 2430	2240 1728	120	684 516	870 628	448 260
平 均	2853	2711	1984	平 均	600	749	351
60	2128 1582	2171 1737	1251 1053	360	306 124	471 231	157 99
平 均	1855	1954	1102	平 均	215	351	123

単位 = cpm/mg N

図4 LiA-³⁵Sの肝Mit 蛋白への取りこみ (in vivo)



ように著明な差は認められなかった。

(4) LiA-³⁵S 投与後の肝 Mit 内結合型 LiA の経時的变化

大量投与2週目のII, IXにLiA-³⁵Sを20mg/kg投与内に投与し肝Mit蛋白へのとりこみを経時的にみた結果表3, 図4に示すように30分後ではB₁群は対照とはほぼひとしいとりこみをみたが、B₆群は対照に比して70%にすぎなかった。しかしいずれも急速に減少し6時間後にはほとんど対照との差が認められなくなった。

表4 蛋白質結合型LiA 合成能へのB₁, CoC, B₆, PAL-P添加の影響

添 加	cpm/mg N
—	3950
B ₁	3797
CoC	3885
B ₆	3416
PAL-P	4070
B ₁ , CoC $2 \times 10^{-5} M$	B ₆ , PAL-P $4 \times 10^{-7} M$

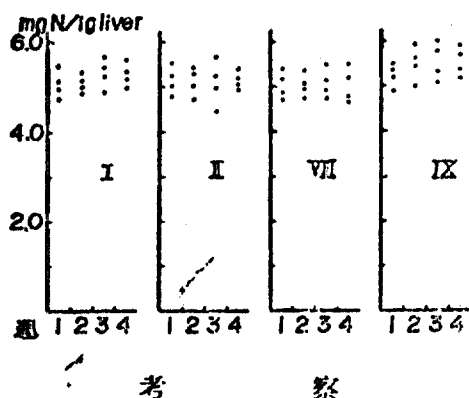
(5) 添加実験

対照群の肝 Mit に Bi , $\text{CoC}^{*5)}$, B_6 , $\text{PAL-P}^{*6)}$ を *in vitro* で添加しその合成能にたいする影響をみたが表 4 に示すように、いずれも無添加のものと有意の差はなかった。

(6) 肝 Mit 窒素量

図 5 にみるように各群とも有意の差は認められなかった。

図 5 肝 Mit-N 量



最近のビタミン化学の進歩、とくにB群化合物の生理作用や代謝作用にかんする研究の発展とあいまって、いわゆる“活性型”、“補給表型”または“体内維持型”、“吸収促進型”と称する種々の製剤をうみだし、しかも本来のビタミンとしての機能よりも薬理的効果を期待して大量の投与が各臨床分野で行なわれるようになった。とくに Bi にかんしてわが国の生化学、薬学、臨床医学における研究はめざましくわれわれ小児科学の領域でも各種神経・筋疾患、栄養障害、リウマチ性疾患、自律神経不安定症候群など Bi あるいはその誘導体の大量投与の適応の範囲はいちじるしく拡大している。しかし一方ではわが国の研究者により、とくに Bi の大量投与時に B_6 をはじめとする他のB群ビタミンの排泄増加や附リン障害を来すとの主張もあり、このようなある特定のビタミンの大量投与により他のビタミンの利用障害その他生化学的前作用というべき現象がどの程度、またどのような機序でいかなる面におこってくるのか、できるだけ早く充分検討することが必要である。

著者はこの線にそつて上述のような実験を行ない

定の成績を得た。すなわち Bi-HCl を幼シロネズミに1日体重1kgあたり1.10および50mgを4週間毎日投与したところ1~2週間は1日20~40mgの Bi を与えた対照に比べて体重増加、肝 Mit における前立結合型 LiA の生成ともに明らかに促進されたが3~4週目になると大量投与を続けているにもかかわらず体重、結合型 LiA 合成能ともに対照と同等となった。 TTFD を上記 Bi-HCl と等価になるように大量投与したはい1mg、10mg/kg Bi-HCl 相当量の投与では Bi-HCl と同様に体重増加、結合型 LiA 合成能ともにあきらかに促進されたが50mg/kgを投与すると1~2週間で体重増加は対照よりわるくなり、かつ結合型 LiA 合成能は対照とほとんど変わらなかった。他方、 B_6 の1日体重1kgあたり1mgの投与では4週間目まで体重増減、結合型 LiA 合成能ともに影響がなく、 B_6 の同量投与では体重増加は対照よりわるく結合型 LiA の生成は1~3週間で低下することが観察された。しかし投与のこのような影響も連続投与しても4週目ではほとんど消失し、体重、結合型 LiA 合成能は対照と変わらない程度まで回復した。

以上の成績を総合的にながめると影響のまつたくみられなかった B_6 はべつとして Bi または B_6 の大量投与はともに体重と結合型 LiA 合成能を平行して変化させたことが注目される。のみならずこれらの変化は Bi または B_6 をひきだいて投与してもまもなく消失していつたから Bi , B_6 の大量投与により生体の発育、代謝系は体重増加にまで変化のおよぶかなり広範な影響をうけ二次的に結合型 LiA 合成能に促進または阻害の効果が現われたものと考えらるべきであるが、その投与を続けていくうちに生体は影響された代謝の立て直しを行なうか、または大量の Bi , B_6 を処理する機構たとえば遠慮酵素系の生成などによつてこれらの変化を消失せしめる可能性も推測することができる。なお結合型 LiA の生成にあらわれた変化が Bi , CoC , B_6 , PAL-P などの直接効果でないことは対照群の肝 Mit にこれらを直接添加してもなんらの影響をしめさなかつた事実より明らかである。

さて Bi 大量投与によつて生体内でどのような代謝上の変化がおこつたかは詳細に検討されていないのでここでは具体的に議論することはできない。はじめに述べたように Bi を大量に与えると長時間後に B_6 の排泄増加の起こることが Klapp⁹⁾により認められ、これと前

*5) CoC =Coccarboxylase*6) PAL-P =Pyridoxal phosphate

後して井上²³⁾は臨床的に TTFD を使用した患者に B₁₂ 欠乏症状のあらわれることを、品川²⁴⁾は B₁₂, TAD²⁵⁾ の大量投与により尿中 B₁₂, ニコチン酸の著明な排泄増加のおこることを経験している。またこれより数年早く Cherillard²⁶⁾ は B₁₂ の附リン反応が B₆ や B₁₂ で阻害されることを述べ等量の B₆ は B₁₂ のエステル化を完全に阻害するとし一方、大量の B₁₂ の存在下では逆に B₆ の附リンが障害されることをみている。さらに Richards²⁷⁾ は幼シロネズミに B₁₂ の大量を投与すると B₆ の欠乏症状が発現し、ついで他のビタミン不足がおこることを記載しており、これら諸家の成績は附リンの反応をめぐって B₁, B₆, B₁₂ のあいだに複雑な競合現象のあることを示唆しているものと考えられる。しかし B₁₂ の投与量を種々変えて動物実験を行なった村田らの実験では B₁₂ を大量摂取しても臓器内 B₁₂ 量に大きな変化が認められていない。しかしいずれにせよこれらの諸説は B₁₂ 大量投与時の代謝にかんする研究の主要なものであるが著者が本報に述べた実験結果の解釈にたいする直接のてがかりを与えるものとは考えられない。

さきに教室の壬生²⁸⁾ は食餌中のアミノ酸または蛋白質の組成が結合型 LiA の生成に大きな影響を与えることを報告した。したがって B₁₂ の大量投与が食餌中の蛋白質利用に影響し、このことを通じて結合型 LiA の生成を左右する可能性を考えたが、著者の測定した肝 Mit 酵素量では各群間に特別の差はなく量的な把握を見出すことはできなかった。なお投与量を 50mg/kg としたばあい B₁₂ と TTFD とでは異なる結果を示したことも明らかな事実であるが、その説明は困難である。TTFD の腸管よりの吸収は B₁₂-HCl よりも良好であるから体内にとりいれられるときの濃度勾配は当然 B₁₂-HCl より高いはずである。このような差があいついたばあい著者のような成績の差となつて現われるのか、あるいは体重、結合型 LiA 合成能におよぼす効果にたいして B₁₂ 投与量に至遠範囲があるのか、それとも B₁₂, TTFD の構造上の違いが大量の投与によつて上述のような薬理効果に差をきたしたものは今後にのこされた課題である。

最後に B₁₂ 大量投与時に体重増加、結合型 LiA 合成能のいずれにもよくない影響があつたことは上述のようであるが、このばあい生体内のいかなる代謝系に一次的に変化がおこるかという点はまだ詳細な報告がない。さきに山田²⁹⁾ は B₁₂, B₆ およびそれらの誘導体 50mg

を静注すると TTFD では 2 時間後に血中インシュリン活性の上昇、血糖値の低下を、B₆, PAL-P でも血中インシュリン活性の上昇をきたすことを認めており、また下重体、副腎切除シロネズミにたいしても TTFD, B₆, PAL-P の腹腔内注射時は血糖下降をきたすことを見出している。さらに山田はヒトに 100mg の TTFD または B₆ を静注し 1 時間後に尿中に 17-OHCS の排泄量がふえることを報告している。このような成績からみると B₁₂ 大量投与は腸管、肝その他の種々の内分泌系にたいして影響を与えているようで、このような全身的影響が体重の減や LiA の活性化反応などにも二次的におよぶことは、いちおう考慮に値することであるが詳細は今後にのこされた問題である。

また B₁₂ 大量投与 2 週間目のシロネズミに LiA-MS を注射し *in vivo* における結合型 LiA 生成をみた結果、対照より著明な低値をしめしたことは表 3, 図 4 の通りである。このばあいに述べた Cherillard²⁶⁾ の主張のように体内での B₆ のリン酸化が結合型 LiA 生成に必要な ATP 依存の反応と競合している可能性も考えられる。なお本報で述べた B₁₂, B₆ 投与の成績をみると、その効果はいずれにしても一時的であつて臨床応用に徒らな長期間の適用は無意味のばあいもあることを示唆するものであろう。

以上 3 報を通じて著者は各種水溶性ビタミンの存在と結合型 LiA の生成、維持との相互関係を検討した対象となるビタミンの種類、投与量によつて種々異なつた、しかも特徴ある結果の得られることを知つた。このような結果のよつてくるところはまだ不明の点が多いが、その実験内容はわれわれの臨床にあたつていまだちにつながりをもつものであるから、これをもとにしてさらに詳細な機序の解明と臨床面への正しい適応に心がけなければならないと思う。

結 論

幼シロネズミに B₁₂-HCl, TTFD, B₆, B₁₂ の大量経口投与を行ないつぎの成績を得た。

- 1) B₁₂-HCl 投与時にはその量に関係なく蛋白質結合型 LiA 合成能の増大を認めた。
- 2) TTFD のばあい体重あたり 1 および 10mg/kg 投与では B₁₂-HCl 投与時と同じ態度をしめしたが 50mg/kg 投与では結合型 LiA 合成能は低下した。
- 3) B₆ 大量投与は蛋白質結合型 LiA 合成能になんら

²⁵⁾ TAD = Thiamine allyldisulfide

の影響をおよぼさなかつた。

4) B₆の大量投与は一時的に蛋白結合型 LiA 合成能が低下した。

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Kobayashi, G.: EFFECTS OF VITAMIN B₁ AND THIAMINE PROPYLDISULFIDE (TPD) ON THE HEART AND KIDNEYS OF CHICKEN EMBRYOS. Nippon Yakurigaku Zasshi, Vol. 56, pp. 959-970, 1960. Faculty of Pharmacology, Showa Medical College, Tokyo (Director, Prof. Kadoo)

INTRODUCTION

The effect of vitamin B₁ on the embryonic stage of development has been studied from various angles by different investigators. Nishio et al. (1,2) studied the effect of B₁ on the growth of human fetus, Ida et al. (3) investigated the distribution of B₁ in internal organs in human fetuses, and Nishizawa (4) and Fujita et al. (5) followed the changes in B₁ concentration in chicken embryos during the incubation stage. Kimura et al. (6) studied the effect of B₁ on the tissue culture of chicken embryo.

At this faculty, Hayashida (7) carried out an experiment in which B₁ and thiamine propyldisulfide (TPD) were administered to fertilized chicken eggs, and their effect on the growth of embryo during the incubation process and the fate of B₁ in the chicken embryos were investigated. B₁ and TPD produced no significant change in the general growth of chicken embryos, but at large doses, growth retardation was noted, with TPD being slightly stronger than B₁. The amount of B₁ excreted into the allantoic fluid and the liver B₁ level also increased upon B₁ and TPD loading, and the excretion of B₁ was particularly notable after the administration of TPD. Asai (8) administered thiamine allyldisulfide and B₁, and found that the compounds exerted no significant effect on the general growth of chicken embryos, but retarded their general growth at large doses and increased the excretion of B₁ into the allantoic fluid and the B₁ level in the liver. He also reported that the amount of B₁ excreted during the late incubation stage was small when TAD was administered, whereas the liver B₁ level rose upon loading of TAD. Murozuka (9) studied the relationship between B₁ metabolism and nicotine, and published a report on the excretion of B₁ into the allantoic fluid and B₁ liver level. Later, Kobayashi (10) carried out a similar study in which the effect of glucuronic acid on B₁ metabolism following the administration of B₁ and TPD was studied, and published its results of considerable interest.

On the other hand, there are relatively few papers concerning the histologic changes in various organs as a result of B₁ administration, and this can be attributed to the fact that, in many cases, large dose administration of B₁ rarely causes any side effect in human. Watanabe (11) reported the condition of the liver of chicken embryo as a result of the injection of B₁ and TPD into fertilized chicken eggs, and Takayanagi (12) discussed the histological image of the liver, kidneys, and heart following the administration of B₁ and TAD.

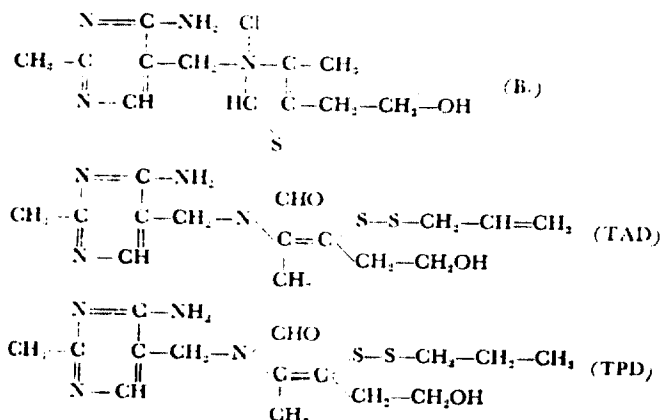
In this experiment, B₁ and TPD were injected into fertilized chicken eggs and their effects on the heart and kidneys of chicken embryos during the incubation period were histologically studied. In the following discussion, the results will be compared with the findings obtained by other investigator.

EXPERIMENTAL MATERIALS AND PROCEDURE

Fresh white Leghorn chicken eggs weighing approximately 50 g were used as experimental materials. After deformed or irregular eggs were removed, seemingly normal eggs were placed in an automatic control electric plain incubator installed in this faculty. The incubation temperature was 101-102°F (top) during the first half, and 102-103°F during the second half of incubation period. The humidity was controlled within a range of 60 - 70%. The ventilation was properly controlled according to the growth of the eggs. The eggs were turned over twice daily, in the morning and in the evening, and examined on the 5th day of incubation, at which nonfertilized eggs and dead eggs were discarded. Dead eggs were again eliminated on the 12th, 15th, and 18th days, and 5 embryos from each group, randomly selected, were subjected to the experiment.

The drugs, B₁ hydrochloride and TPD, were mixed in gum arabic and the solution was emulsified with distilled water. The amount injected was adjusted so that 0.1 cc portions of the emulsion contained 1, 3, 5, 8, 10, 13, 15, and 18 mg of the drugs. The amount of injection was 0.1 cc per egg. For the control, a gum arabic emulsion (gummi arabicum, 5 mg/0.1cc) was given at 0.1 cc per egg. In the injection of the emulsions, the pointed end of the egg was cleaned with disinfectant alcohol and a small hole was drilled. The required amount of emulsion was injected into the egg white with a 1/2 injection needle and a tuberculin syringe, while care was taken not to spill the egg white or the emulsion. The hole was sealed with paraffin, and the egg was placed in the incubator. This procedure was carried out under aseptic condition. The number of eggs used was 100 per stage, and 80 eggs were used as the control. In the preparation of tissue specimens, the heart and kidneys of the embryos were removed, fixed with formalin, imbedded in paraffin, and stained with hematoxylin-eosin or Azan Mallory. Frozen sections were also prepared and fat-stained with Sudan III. In the examination of the kidneys, the hind kidney was used.

The structural formulas of B₁ and TPD are given below:



A. HEART

I. 12TH DAY OF INCUBATION

1. CONTROL

The pericardium was separated in some areas. There was no sign of atrophy but slight cellular infiltration was noted in some areas. One case indicated an aggregation of cells appearing as endothelial cells which might be the embryonic enlage of the blood vessels surrounding the origin of an artery of the adventitial coat. The pericardial vessels exhibited mild distention. The myocardial layer exhibited no interstitial infiltration of cells, but the cardiac muscle itself was composed of extremely fine muscular fibers, some running in a reticular fashion and some closely together. There were several stationary blood image in the space among the fibers. There was no hemmorhagic sign or proliferative change of connective tissues. A large proportion of the endocardium was separated, and the remaining segment indicated neither enlargement nor proliferation of endothelial cells. The subendocardial tissue also showed no cellular infiltration or edema.

2. B₁ GROUPS

At 1 mg, some areas of the pericardium indicated mild cellular infiltration, and mild distention of pericardial vessels was noted. The cardiac muscle itself appeared same as that of the control, with mild distention of some of the vessels in the myocardial layer. One case exhibited a mild proliferative image of endothelial cells of the endocardium. The 3 mg group indicated cellular infiltration and distention in the pericardium to the same degree as the 1 mg group. There was also mild distention in the myocardial layer. The proliferation of endothelial cells in the endocardium was shown only by 1 case, just as in the control group, and slight enlargement of endothelial cells was noted in all cases. The 5 mg group was marked with mild infiltration of round cells in the interstice of the cardiac muscle, and vascular distention and mild cellular infiltration in the perivascular regions in the myocardial layer were noted. One case indicated proliferation of endothelial cells in the endocardium. The 8 mg group exhibited mild cellular infiltration of the pericardium, pronounced vascular distention in the myocardial layer, and slight enlargement and proliferation of endothelial cells in the endocardium. One of the cases displayed mild edema in the subendocardial tissue. Just as the 8 mg group, the 10 mg group exhibited mild vascular distention in the myocardial layer. Hemorrhagic image was also shown by 1 case, but was only of mild severity. The 13 mg group exhibited mild cellular infiltration and vascular distention in the pericardium, and slight interstitial infiltration of round cells. The cardiac muscle itself exhibited nuclear concentration in one case, and the blood vessels in the myocardial layer also indicated distention just as the pericardium, and cellular infiltration in the perivascular region. Furthermore, the endocardium exhibited a proliferative image of endothelial cells, and 1 case revealed slight enlargement of endothelial cells. One case revealed cellular infiltration

in the subendocardial tissue. The 15 mg group exhibited slight vascular distention and cellular infiltration in the pericardium, and vascular distention in the myocardial layer. The 18 mg group only indicated vascular distention of the myocardial layer and cellular infiltration in the surrounding area. They showed no other change.

TABLE 1

[illegible]

KEY: a, histologic impression of the heart; b, incubation days; c, material given; d, control; e, gum arabic; f, day; g, pericardium; h, cellular infiltration; i, vascular distention; j, myocardial layer; k, interstitial infiltration of cells; m, enlargement; l, cardiac muscle; n, atrophy; o, fatty degeneration; p, necrobiosis or necrosis; q, blood vessels; r, distention; s, hemorrhage; t, proliferation of connective tissue; u, endocardium; v, endothelial cell; w, proliferation; x, subendocardial tissue; y, edema.

KEY: a, histologic impression of the kidneys; b, incubation stage; c, material given; d, control; e, gum arabic; f, day; g, covering; h, glomeruli; i, enlargement; j, atrophy; k, hyaline degeneration; l, fibrosis; m, destruction of Henle's loop; n, hyperemia; o, Bowman's capsules; p, dilation; q, adhesion; r, hemorrhage; s, granular degeneration; t, renal tubules; u, loss of nucleus; v, fat; w, vacuolation; x, interstice; y, hyaline casts; z, necrobiosis or necrosis; a', dilation of lumen; b', narrowing of lumen; c', interstitial infiltration of cells; d', blood vessels; e' hyperemia and congestion; f', perivascular infiltration of cells; g', mucous membrane of the pelvis of the kidney.

3. TPD GROUPS

In the 1 mg group, mild vascular distention and cellular infiltration of pericardium were present, and interstitial infiltration of round cells was also noted. One case indicated nuclear concentration in the cardiac muscle itself, and another case, localized necrobiosis, circulatory disturbance of peripheral vessels, and pronounced vascular distention in the myocardial layer. The 3mg group indicated mild vascular distention in the pericardium and the myocardial layer. The endocardium revealed mild proliferation of endothelial cells in some areas. The 5 mg group showed mild cellular infiltration in the pericardium, and the perivascular regions of the myocardial layer. The endocardium exhibited enlargement of endothelial cells. The 8 mg group revealed mild vascular distention in both the pericardium and the myocardial layer, and infiltration of round cells in the perivascular region of the myocardial layer. The 10 mg group only showed mild vascular distention in the myocardial layer. The 13 mg group indicated mild cellular infiltration in the pericardium and vascular distention in the myocardial layer. The 15 mg group occasionally gave signs of myocardial necrobiosis and peripheral circulatory disturbance. The myocardial layer showed vascular distention and mild cellular infiltration in the surrounding area. The 18 mg group exhibited mild cellular infiltration in the pericardium, and peripheral circulatory disturbance and pronounced vascular distention in the myocardial layer. The cases additionally exhibited proliferation of the connective tissue.

II. 15TH DAY OF INCUBATION

1. CONTROL

The pericardium revealed mild cellular infiltration and vascular distention, and there was mild interstitial cellular infiltration. The cardiac muscle itself showed slightly thicker fibers as compared to that of the 12th day group, but it had not yet developed the striated pattern. The myocardial layer displayed a somewhat prominent image of vascular distention, with mild general cellular infiltration surrounding the vessels. The subendocardial tissue was normal but 1 case clearly showed enlargement and proliferation of endothelial cells.

2. B₁ GROUPS

In the 1 mg group, the blood vessels in both pericardium and myocardial layer appeared distended, and there was mild interstitial infiltration of round cells. The endocardium revealed enlargement and proliferation of endothelial cells in some cases. The 3 mg group showed mild infiltration of round cells and vascular distention in the pericardium and myocardial layer. The 5 mg group exhibited mild vascular distention and infiltration of round cells in the pericardium and myocardial layer, and some areas of the myocardial layer exhibited a hemorrhagic image. Furthermore, the endocardium revealed slight enlargement of endothelial cells. In the 8 mg group, mild cellular infiltration and vascular distention were present in the pericardium and myocardial layer, and the endothelial cells in the endocardium had become enlarged. The 10 mg group indicated mild cellular infiltration in the pericardium, and vascular distention in the myocardial

layer, with cellular infiltration in the surrounding area. The 13 mg group revealed vascular distention and cellular infiltration in the pericardium and myocardial layer, and proliferation of connective tissue in the myocardial layer. The endothelial cells in the endocardium were slightly enlarged and proliferated. The 15 mg group showed mild cellular infiltration and vascular distention in the pericardium, and vascular distention in the myocardial layer. The endocardium was marked with slightly enlarged endothelial cells. The 18 mg group showed interstitial infiltration of cells to a slight degree, and vascular distention in the myocardial layer, with mild infiltration of round cells around the vessels. There was also mild cellular infiltration in the subendocardial tissue.

3. TPD GROUPS

The 1 mg group revealed mild vascular distention in the pericardium and myocardial layer, and mild infiltration of round cells in the interstice and myocardial layer. In the endocardium, some of the endothelial cells were enlarged. The changes shown by the 3 mg group were similar to those of the 1 mg group, with perivascular infiltration of cells in the myocardial layer. The 5 mg group also exhibited changes similar to those of the 1 mg group. The 8 mg group revealed mild vascular distention and pronounced cellular infiltration in the pericardium. The myocardial interstice also indicated mild cellular infiltration. In the myocardial layer, peripheral circulatory disturbance was visible in some of the cases. The vessels of the myocardial layer were mildly distended and round cells had infiltrated the perivascular regions. In the 10 mg group, round cell infiltration and vascular distention marked the pericardium, and mild vascular distention, the myocardial layer. In some of the cases, the endocardium showed enlargement and proliferation of endothelial cells. The 13 mg group only exhibited a localized necrotic change in one area of the myocardial layer. The 15 mg group exhibited vascular distention in the myocardial layer, and peripheral circulatory disturbance and necrobiosis of cardiac muscle in some of the cases. The 18 mg group showed localized necrotic image in a part of the myocardial layer, and pronounced vascular distention. Round cells have mildly infiltrated in the perivascular regions, with proliferation of connective tissue in some cases. Endothelial cells were enlarged in some places of the endocardium.

II. 18TH DAY OF INCUBATION

1. CONTROL

The pericardium showed mild round cell infiltration, but no vascular distention. There was no interstitial infiltration of cells in the myocardial layer. The cardiac muscle itself became thickened from the 12th to the 15th day, occasionally displaying a striated pattern. The myocardial layer showed mild vascular distention, but the endocardium indicated no change at all.

2. B₁ GROUPS

In some cases of the 1 mg group, the pericardium was thickened. The blood vessels were mildly distended and the interstice showed cellular infiltration. The 3 mg group merely showed cellular infiltration in the

pericardium and vascular distention in the myocardial layer. The 5 mg group showed peripheral circulatory disturbance in some parts of the myocardial layer, which also exhibited mild vascular distention. The pericardium of the 8 mg group revealed cellular infiltration and vascular distention, and some areas of the myocardial layer were marked with fragmentation of muscle. There were mild vascular distention and cellular infiltration in the myocardial layer, and the tissue below the inner coat of some parts of the endocardium was edematous. The pericardium of the 10 mg group revealed mild cellular infiltration and vascular distention, and slight interstitial infiltration of cells. The myocardial layer exhibited peripheral circulatory disturbance and hemorrhagic image in some places. Mild interstitial infiltration of cells was also noted. In some areas of the myocardial layer, peripheral circulatory disturbance and hemorrhagic image were present. The 13 mg group showed no change other than mild vascular distention in the myocardial layer. The 15 mg group exhibited infiltration of round cells in the pericardium and interstice, and vascular distention of low severity in the pericardium and myocardial layer. In the endocardium, the endothelial cells were partially enlarged to a slight degree. The 18 mg group only showed vascular distention in the pericardium, with no other noteworthy change.

3. TPD GROUPS

The 1 mg group showed vascular distention in the pericardium, and interstitial infiltration of cells. Some parts of the myocardial layer revealed peripheral circulatory disturbance. Some cases of the 3 mg group showed the thickening of the pericardium. There were also mild interstitial infiltration of cells and the enlargement and proliferation of endothelial cells in the endocardium. The 5 mg group also showed partial thickening of the pericardium with cellular infiltration in the adventitial coat. The myocardial layer revealed peripheral circulatory disturbance and vascular distention. The 8 mg group showed vascular distention in the pericardium and myocardial layer, and cellular infiltration marked the perivascular regions in the pericardium and myocardial layer. Interstitial infiltration of cells was also present. Furthermore, some areas of the myocardial layer revealed peripheral circulatory disturbance, and the muscle itself occasionally exhibited cloudy swelling. The muscular layer of the 10 mg group showed mild interstitial infiltration of cells, and vascular distention with cellular infiltration in the surrounding area. Some areas of the myocardial layer revealed peripheral circulatory disturbance, and the endocardium, mild proliferation of endothelial cells in some places. The pericardium of the 13 mg group indicated mild vascular distention, and the myocardial layer exhibited interstitial infiltration of cells and peripheral circulatory disturbance. The cardiac muscle had partially developed cloudy swelling, and the blood vessels in the myocardial layer were distended, with round cell infiltrating in the perivascular region. Some of the endothelial cells in the endocardium were enlarged. The 15 mg group showed mild interstitial infiltration of cells, with peripheral circulatory disturbance. Some areas of the myocardial layer indicated localized necrobiotic or necrotic changes, with vascular distention and perivascular infiltration of cells. In the endocardium, the endothelial cells were enlarged, with proliferative signs. The 18 mg group showed pericardial vascular distention, and interstitial infiltration of cells. The myocardial layer contained mild, localized necrotic image, with vascular distention and mild cellular infiltration in the perivascular regions.

B. KIDNEYS
(Tables 3 and 4)

I. 12TH DAY OF INCUBATION

1. CONTROL

The covering was almost completely separated and not visible. The glomeruli were irregular in size, but there was no adhesion with Bowman's capsules. The renal tubules showed partial nuclear diminution. The interstice indicated vascular congestion and hyperemia, and mild cellular infiltration in the surrounding area. The pelvis of the kidney contained no mucous membrane.

2. B₁ GROUPS

The 1 mg group showed edematous enlargement of some glomeruli, and a small amount of hyaline casts were present in some areas of the renal tubules. There were mild cellular infiltration in the perivascular regions and vascular congestion in the interstice. The 3 mg group showed generally enlarged glomeruli, with some of them adhering to Bowman's capsules. The renal tubules indicated nuclear loss and presence of some hyaline casts. The interstice revealed cellular infiltration. The interstitial blood vessels were mildly congested. The 5 mg group showed granular degeneration, nuclear diminution and hyaline cast formation in renal tubules. The interstitial blood vessels were severely congested. The 8 mg group also exhibited nuclear diminution and congestion of interstitial blood vessels. In the 10 mg group, the glomeruli were generally large and were adhering to Bowman's capsules in some places. The renal tubules were generally dilated with mild changes such as nuclear loss and vacuolation of tubular wall. The 13 mg group showed glomerular degeneration and diminution of nucleus in the renal tubules, and tubular dilation in some cases. The interstitial vessels were congested. In the 15 mg group, the glomeruli were generally enlarged and some of them exhibited hyperemic image. Some of the renal tubules were dilated, with mild granular degeneration and vacuolation. In the 18 mg group, some glomeruli were swollen, with hyperemic image. The lumen of renal tubules was slightly dilated, with occasional granular degeneration and hyaline cast formation. There were mild necrobiosis and congestion of interstitial vessels.

3. TPD GROUPS

Some of the 1 mg group indicated adhesion between Bowman's capsules and the glomeruli. The renal tubules contained hyaline casts. The interstitial vessels were slightly congested. In the 3 mg group, edematous enlargement of the glomeruli was noted just as in the 1 mg group, and the lumen of some of the renal tubules was dilated. Furthermore, there were hyaline casts and signs of granular degeneration in a part of the renal tubules. The interstitial vessels were mildly congested. Some cases of the 5 mg group exhibited edematous enlargement of the glomeruli, and atrophied glomeruli although the proportion of such glomeruli was small. The renal tubules partly indicated granular degeneration and hyaline cast formation. The interstitial blood vessels were congested. Some of the 8 mg group indicated glomerular hyperemia, and occasional signs of mild granular degeneration in the renal tubules. The interstitial blood vessels

were mildly congested. The 10 mg group also indicated some cases with glomerular enlargement and, in some cases, congestion as well. The renal tubules exhibited mild granular degeneration, and the interstitial blood vessels were congested. The 13 mg group indicated mild enlargement of the glomeruli, and the lumen of some of the renal tubules was dilated. Mild granular degeneration and hyaline cast formation were also present. In the 15 mg group, some of the renal tubules exhibited granular degeneration and hyaline cast formation, and the interstitial blood vessels appeared congested. The 18 mg group showed edematous enlargement of some of the glomeruli, with partial destruction of Henle's loops. Some cases showed enlargement of Bowman's capsules. Tubular lumen was slightly dilated, and granular degeneration and hyaline cast formation were noted in some cases.

TABLE 3

c 投与物質		T P D																																			
		1 mg				3 mg				5 mg				8 mg				10mg				13mg				15mg				18mg							
		12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18			
心臓組織学的所見																																					
心外膜	f 細胞浸潤	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
	g 血管充満	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
心筋	h 心臓組織学的所見																																				
	i 肥厚	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
	j 萎縮	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
	k 脂肪	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
筋自身	l 類癌死〜壊死	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
血管	m 細胞浸潤	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
	n 血管充満	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
出血	o 出血	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
結合	p 結合組織増加	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
心臓内	q 心臓組織学的所見																																				
	r 肥厚	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
心臓内	s 増大	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
	t 心臓組織学的所見																																				
心臓内	u 水腫	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-				
	v 心臓組織学的所見																																				
心臓内	w 心臓組織学的所見																																				

KEY: a, histological impression of the heart; b, incubation stage; c, material given; d, day; e, pericardium; f, cellular infiltration; g, vascular distention; h, myocardial layer; i, interstitial infiltration of cells; j, cardiac muscle; k, enlargement; l, atrophy; m, fat; n, necrobiosis or necrosis; o, blood vessels; p, hemorrhage; q, proliferation of connective tissue; r, endocardium; s, endothelial cells; t, subendocardial tissue; v, edema

TABLE 4

C 投与物質			T P D																															
			1 mg				3 mg				5 mg				8 mg				10 mg				13 mg				15 mg				18 mg			
腎臓組織学的所見			12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18		
f	系	被 c 膜																																
		順 g 大																																
		萎 h 縮																																
		硝子様化																																
		球 纖 維 化																																
m	ボーマン氏囊	糸球体の崩壊																																
		血 l 充																																
		膜 n 膨																																
g	腎 臓	萎 o 着																																
		血 p 出																																
		粒 r 変性																																
		核 s 消 失																																
		脂 t 肪																																
u	尿	管 u 腔 變 化																																
		管 v 腔 再 生																																
		管 w 壊死-壊死																																
x	腎 臓	腔 x 腔 膨																																
		腔 y 腔 小																																
		腔 z 腔 變 化																																
a'	腎 臓	血 a' 血 管 炎																																
		血 b' 血 管 炎																																
		血 c' 血 管 炎																																

KEY: a, histologic image of the kidney; b, incubation stage; c, material given; d, day; e, covering; f, glomeruli; g, enlargement; h, atrophy; i, hyaline degeneration; j, fibrosis; k, destruction of Henle's loops; l, hyperemia; m, Bowman's capsules; n, enlargement; o, adhesion; p, hemorrhage; q, renal tubules; r, granular degeneration; s, loss of nucleus; t, fat; u, vacuolation; v, hyaline casts; w, necrobiosis or necrosis; x, luminal enlargement; y, narrowing of lumen; z, interstice; a', interstitial infiltration of cells; b' blood vessels; c', hyperemia and congestion; d', perivascular infiltration of cells; e', mucous membrane of pelvis

II. 15TH DAY OF INCUBATION

1. CONTROL

As compared to the 12th day group, the glomeruli were generally atrophied, some adhering to Bowman's capsules. In all cases, the renal tubules showed the diminution of nucleus to a slight degree. The interstitial blood vessels were notably congested.

2. B₁ GROUPS

The only change shown by the 1 mg group was mild changes in the renal tubules, i.e., diminution of nucleus, but the 3 mg group also showed granular degeneration. The renal tubules in the 5 mg group indicated mild granular degeneration and nuclear diminution, with hyaline casts in some cases. Tubular lumen was partially narrowed. The 8 mg group indicated overall atrophy of the glomeruli, with tubular lumen also narrowed in some areas and mild necrobiosis. The 10 mg group occasionally indicated shrinking of the glomeruli, and mild granular degeneration and vacuolation in the renal tubules. Some cases exhibited nuclear diminution. The 13 mg group indicated overall reduction in size of the glomeruli, just as the 10 mg group, and tubular lumen was narrowed to some degree, with granular degeneration in some instances. Interstitial blood vessels were also congested. The 15 mg group was marked with partial edematous enlargement of the glomeruli and slight distention of interstitial blood vessels. The 18 mg group indicated atrophy of some of the glomeruli, and occasional adhesion between Bowman's capsules and the glomeruli. In the interstice, the connective tissue had proliferated, and the blood vessels were mildly congested.

3. TPD GROUPS

The 1 mg group showed swelling of some glomeruli, and occasional adhesion between the glomeruli and Bowman's capsules. Tubular lumen was occasionally narrowed, with slight granular degeneration and hyaline cast formation. Congestion of interstitial vessels was also noted. In some cases of the 3 mg group, the glomeruli were enlarged and the renal tubules had undergone granular degeneration, and the nuclei had disappeared. Some indicated localized necrotic or necrobiotic image. Mild congestion of interstitial blood vessels was also present. The 5 mg group revealed adhesion between Bowman's capsules and the glomeruli, and the granular degeneration and nuclear diminution in the renal tubules were more notable than in the 3 mg group. The necrobiotic image was present in some cases. The interstice was characterized by congestion and hyperemia, and slight cellular infiltration in the surrounding area. The glomeruli of the 8 mg group were irregular in size, with destruction of Henle's loops in some instances. The interstitial blood vessels appeared congested. The 10 mg group showed some atrophied glomeruli, but no change in the renal tubules or interstice. In the 13 mg group, the glomeruli were atrophied in some cases, and tubular lumen was narrowed due to granular degeneration. The 15 mg group showed granular degeneration and hyaline casts in some of the renal tubules, and mild distention of interstitial blood vessels. The 18 mg group indicated atrophy of a part of the glomeruli, and granular degeneration, nuclear diminution, and hyaline cast formation in the renal tubules. There were extremely mild necrobiotic signs in some areas.

II. 18TH DAY OF INCUBATION

1. CONTROL

The glomeruli were uniform in size. There was neither adhesion nor hemorrhage in Bowman's capsules. The renal tubules exhibited partial granular degeneration, vacuolation, and diminution of nucleus. In some areas, tubular lumen was narrowed. No change was noted in the interstice.

2. B₁ GROUPS

In the 1 mg group, Henle's loops were destroyed in some cases, and the renal tubules indicated pronounced granular degeneration and diminution of nucleus. Some of them indicated necrobiosis. Fat-stained preparations of interstice revealed sporadic small fat droplets. Interstitial blood vessels were slightly congested. The 3 mg group, just as 1 mg group, indicated narrowing of tubular lumen, and granular degeneration, nuclear diminution, and fatty degeneration of the tubules to a mild degree. Necrobiotic image was present in some areas. The interstice was mildly congested. In the 5 mg group, the glomeruli were partially distended, and the renal tubules exhibited granular degeneration, nuclear diminution, and hyaline cast formation. There was also necrobiosis. The interstice exhibited round cell infiltration and vascular distention of mild severity, and cellular infiltration in the perivascular regions. In the 8 mg group, pronounced atrophy of the glomeruli appeared sporadically. Tubular lumen was slightly narrowed, with mild granular degeneration and nuclear diminution and necrobiotic image in some areas. Interstitial vascular distention of mild severity was also present. The 10 mg group indicated mild granular degeneration, nuclear diminution, and fatty degeneration in the renal tubules, and necrobiotic image in some cases. The interstice revealed cellular infiltration and vascular distention, with round cells infiltrating around the vessels. In the 13 mg group, tubular lumen was narrowed. There were mild granular degeneration, diminution of nucleus, and hyaline cast formation, and necrobiosis over the entire area. The interstice exhibited slight cellular infiltration and congestion. The 15 mg group indicated overall atrophy of the glomeruli. The renal tubules exhibited granular degeneration, nuclear diminution, vacuolation, and fatty degeneration to mild degrees, with occasional necrobiosis. Tubular lumen was narrowed. The 18 mg group indicated slight atrophy of the glomeruli, and partial adhesion in Bowman's capsules. The renal tubules exhibited pronounced granular degeneration, and also diminution of nucleus and vacuolation, with overall image of necrobiosis and necrosis. Tubular lumen was partially narrowed, and the interstice appeared mildly congested.

3. TPD GROUP

The 1 mg group showed mild granular degeneration, diminution of nucleus, and fatty degeneration in the renal tubules, and some indicated necrobiosis. Slight narrowing of tubular lumen was also present. The 3 mg group indicated mild atrophy of the glomeruli, and granular degeneration, and diminution of nucleus of the renal tubules were also noted. The renal tubules also exhibited fatty degeneration and necrobiosis in some cases. The interstitial blood vessels were mildly distended. The 5 mg group showed mild atrophy of the glomeruli, and granular degeneration and fatty degeneration of the renal tubules. The interstitial vascular distention was mild. The 8 mg

group showed partial atrophy of the glomeruli, and degenerative changes such as granular degeneration, fatty degeneration, and necrobiosis in the renal tubules. The interstice exhibited infiltration of round cells and congestion. The 10 mg group showed mild changes, i.e., granular degeneration, fatty degeneration, and diminution of nucleus in some parts of the renal tubules. The interstitial blood vessels were markedly distended. The 13 mg group showed adhesion between Bowman's capsules and the glomeruli, and granular degeneration and fatty degeneration of low severity in the renal tubules. The interstice appeared congested. The 15 mg group were marked with partial atrophy of the glomeruli, mild granular degeneration, diminution of nucleus, and vacuolar formation in the renal tubules, and pronounced interstitial vascular distention. In the 18 mg group, partial destruction of Henle's loops appeared in some of the glomeruli, and granular degeneration, diminution of nucleus, and hyaline cast formation in the renal tubules. The interstitial blood vessels were notably congested just as in the 15 mg group.

SUMMARY

The author injected B₁ and TPD to chicken eggs, and the histologic image of the heart and kidneys of the embryos was investigated on the 12th, 15, and 18th days of incubation.

Prior to this experiment, the author had injected gum arabic to eggs as a control experiment. As a result, the heart of the embryo showed no significant change other than vascular distention in the pericardium and myocardial layer, and mild adventitial and interstitial cellular infiltration on the 12th day. The result was in agreement with the findings in Takayanagi's experiment with gum arabic and Okada's experiment (13) with Ringer's solution or no solution at all. In the kidney, the glomeruli were somewhat enlarged on the 12th day, but were atrophied on the 15th day. The renal tubules indicated loss of nucleus. On the 18th day, the renal tubules indicated granular degeneration and vacuolar formation, which were not included in Okada's report in conjunction with the control experiment using Ringer's solution or no injection at all. Wada stated that, on the 18th day, the control indicated a vacuol-like space in the protoplasm at a site corresponding to the inner coat of tubular cell but no granular degeneration. In Takayanagi's experiment, the renal tubules exhibited granular degeneration on the 12th, 15th, and 18th days after the injection of gum arabic, and loss of nucleus on the 15th day. In other words, the granular degeneration noted in the present experiment resemble Takayanagi's findings, and is also in agreement with the changes appearing in the kidneys after death as reported by Suzuki (14). The fact that the cloudy swelling in the kidneys accompanies no nuclear change, but the protoplasm is generally swollen with microgranules, has been interpreted differently by various investigators, and the nature of such change is yet to be clarified. Kizawa (15) stated that, if the cloudy swelling was caused due to changes after death, changes appeared in the nucleus in many instances, appearing as the so-called cloudy swelling in the epithelium of the main segment of the renal tubule, and the cloudy swelling in the absence of clinical renal dysfunction is not essential degeneration.

Summarizing the changes shown by the B₁ group, the heart exhibited vascular distention of the pericardium and myocardial layer at all doses,

and mild cellular infiltration in the pericardium, interstice, and perivascular regions. At 5 and 10 mg, peripheral circulatory disturbance appeared in a part of the myocardial layer on the 18th day. There was no other noteworthy change. Takayanagi administered B₁ at 4 different doses, 1, 5, 10, and 15 mg, and obtained the following findings. In an early stage of incubation, the pericardium was thickened, and cellular infiltration occurred to the interstice in an early stage at small doses and in a later stage of incubation at large doses. Proliferation of connective tissue occurred in the later stage. These changes were mild, and are generally in agreement with the author's findings. Nishizawa followed the changes in B₁ level during the incubation process, and found that the B₁ content in the heart, liver, and small intestine was minimal in an early stage, but increased with elapsed time during which the organs developed. According to Ida et al., in the newborns, the B₁ organ concentrations vary in a specific order just as in mature adults, i.e., higher in the order of the heart, liver, and kidneys. As stated above, B₁ exhibits unique affinity with the heart. Shiraishi (16) studied histological changes following 60-day consecutive hypodermic injection of 0.5 mg of B₁ hydrochloride to young mice, and reported that there was no change in the heart. The present experiment also gave similar data. In the kidneys, the glomeruli and Bowman's capsules remained the same as those of the control, but the granular degeneration in the renal tubules increased in severity with time, the severity being proportional to the dose. Hyaline casts were also noted to a slight degree in the 5 mg group and other groups as well throughout the period of observation. Granular degeneration or necrobiotic change in the renal tubules appeared from the 18th day of incubation at smaller dose and from an early stage of incubation at larger dose. These changes appear to be a sort of nephrotic degeneration due to a toxin. These results are contrary to the findings published by Shiraishi, but the difference can be attributed to the difference in the sensitivity toward B₁ between mature animals and embryos. Takayanagi reported that there was no change in the B₁ group in an early stage of incubation, but in a later stage, the glomeruli exhibited infiltration of round cells and the renal tubules, necrobiotic or necrotic image. These changes in the renal tubules are similar to the author's findings. According to Shimazono (17), in rats, when the dose of B₁ is increased, the organ B₁ level rises, and the organs showing high B₁ concentration at saturation are the liver, heart, and kidneys, reaching 1000 % or even higher. Thus, it is already known that B₁ can be stored in a considerably large amount in the kidneys as well as in the heart and liver.

In the heart following the administration of TPD, changes consisted of cellular infiltration and vascular distention of the pericardium, and vascular distention and perivascular cell infiltration in the myocardial layer, which were observed throughout the incubation stage. At larger doses, the endocardium showed enlargement of endothelial cells in a later stage of incubation. The myocardial layer exhibited peripheral circulatory disturbance in the small dose groups and mild localized necrobiotic or necrotic image in each stage of incubation at large doses. Such necrotic changes were not shown by any of the B₁ groups and TPD may be more toxic to the heart than B₁. In Takayanagi's experiment, pericardial infiltration of cells and seemingly reversible vacuolar formation occurred in an early stage, and some of the 10 mg group indicated necrobiotic or necrotic change on the 18th day. In his experiment, such changes never occurred to the B₁ group. Thus, the effects of TAD and TPD on the heart seem to resemble.

In the liver of a chicken embryo studied by Watanabe, a TPD injection caused necrobiotic and necrotic changes in the late stage at large doses, and Hayashida's experiment also demonstrated strong growth disturbance by TPD, whereas B₁ exhibited no growth retardation effect. Thus, their findings concerning growth retardation and hepatic and cardiac disturbance due to large dose are generally in agreement with the present findings. Matsukawa and Suzuoki et al. (18) gave B₁ and TPD to rabbits, and studied organ B₁ concentrations and the phosphorylation rate in different organs. They found that there was no appreciable change between the liver, kidney, and brain, but the B₁ level in the heart was highest in the TPD group. Thus, TPD was found to possess stronger affinity toward the heart than B₁. This may be in some way related to the author's findings.

The kidneys exhibited similar image of the glomeruli and Bowman's capsules as those in the B₁ groups. The renal tubules showed granular degeneration at each dose, and hyaline casts were present. However, necrobiosis in the renal tubules was mildly noted on the 18th day only in the 1, 3, 8, and 13 mg groups. Comparing the TPD groups and the B₁ groups, the histological changes in the kidneys were milder in the former. Takayanagi's 1, 5, 10, and 15 mg TAD groups indicated pronounced granular degeneration, diminution of nucleus, and necrobiotic or necrotic changes in the renal tubules. These changes indicate that TAD produces severe morbid changes in the renal tubules than TPD.

In short, the histological changes of the heart and kidneys following the injection of B₁ and TPD were generally of low severity, and the degenerative changes produced at certain doses seem to be reversible.

CONCLUSION

B₁ and TPD were given to fertilized chicken eggs at 8 doses, and the heart and kidneys of the embryos on the 12th, 15th, and 18th days of incubation were histologically examined. The following conclusions were obtained.

1. B₁ produced no change in the heart. In the kidneys, it caused reversible granular degeneration and necrobiotic or necrotic change with loss of nucleus in the renal tubules from an early stage of incubation at large doses and in the later stage at small doses.
2. TPD produced interstitial infiltration of cells in the heart at small doses, necrobiotic or necrotic changes in the myocardial layer in a later stage of incubation at large doses. Endothelial cells were enlarged and proliferated. In the kidneys, the granular degeneration of the renal tubules was noted in an early stage of incubation in the large dose groups, and reversible granular degeneration and necrobiotic or necrotic changes with loss of nucleus, in the renal tubules of the small dose groups in a later stage of incubation.

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EXPLANATION OF PHOTOGRAPHS

Specimens of the Heart of Chicken Embryos (Hematoxylin-Eosin Preparations)

- Figure 1. 12th day of incubation; B₁; 3 mg
Figure 2. 12th day of incubation; TPD; 1 mg
Figure 3. 15th day of incubation; B₁; 3 mg
Figure 4. 15th day of incubation; B₁; 15 mg
Figure 5. 18th day of incubation; B₁; 18 mg
Figure 6. 18th day of incubation; B₁; 18 mg
Figure 7. 18th day of incubation; TPD; 15 mg
Figure 8. 18th day of incubation; TPD; 18 mg

Specimens of the Kidney of Chicken Embryos (Hematoxylin-Eosin Preparations)

- Figure 9. 12th day of incubation; B₁; 15 mg
Figure 10. 12th day of incubation; TPD; 18 mg
Figure 11. 15th day of incubation; B₁; 8 mg
Figure 12. 15th day of incubation; TPD; 1 mg
Figure 13. 15th day of incubation; TPD; 13 mg
Figure 14. 18th day of incubation; B₁; 15 mg
Figure 15. 18th day of incubation; TPD; 15 mg
Figure 16. 18th day of incubation; TPD; 18 mg

Nippon Yakurigaku Zasshi 56:959-970. 1960.

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日薬理誌 56, 959-970 (1960)

Vitamin B₁ 並びに Thiamine Propyldisulfide (TPD) の鶏胎仔心臓、腎臓に及ぼす影響に就いて

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摘 要

Vitamin B₁ (以下 B₁ と略す) 及びその誘導体の胎生期に於ける研究に就いては、西澤等¹⁾が B₁ 投与の胎児の发育に及ぼす影響を、又井田等²⁾は胎児に於ける B₁ 含有量及び B₁ の臓器内での分布状況³⁾を報告している。更に西沢⁴⁾、藤田等⁵⁾は孵化過程に於ける鶏胎仔の B₁ の消長に就いて追討し、木村等⁶⁾は卵黄嚢胚の培養に及ぼす B₁ の影響に就いて記載している。

当教室に於ても、林田⁷⁾は B₁ 並びに Thiamine propyldisulfide (以下 TPD と略す) を有精卵卵黄に投与し、孵化過程に於ける鶏胎仔の发育に及ぼす影響と鶏胎仔体内の B₁ の運命に就いて検討した。即ち B₁ 並びに TPD は鶏胎仔の一般发育には著しい影響を及ぼさないが、大量投与を行うと发育抑制が起り、その傾向は TPD の方が稍々強く、尿囊水中への B₁ 排泄、肝臓内 B₁ 含有量も B₁ 並びに TPD 投与によって増加するが、B₁ の排泄は TPD 投与の場合に概ね著しいことを認めた。次いで浅井⁸⁾は B₁ 及び Thiamine allyldisulfide (以下 TAD と略す) を投与した処、B₁ 並びに TAD は鶏胎仔一般发育に対し著大な影響を及ぼさないが、大量投与による发育抑制の傾向が認められ、尿囊水中への B₁ 排泄、肝臓内 B₁ 含有量は B₁ 並びに TAD 投与により増加するが、孵卵末期に於ける B₁ 排泄量は TAD 投与時に低値を示すが、肝臓内 B₁ 含有量は逆に TAD 投与時が優ると記している。更に室塚⁹⁾は鶏胎仔の B₁ 代謝と Nicotine との関係を検討し、尿囊水中への B₁ 排泄並びに肝臓内 B₁ の濃度を報告している。その後小林¹⁰⁾も同様に鶏胎仔の B₁ 並びに TPD 投与の場合に於ける B₁ 代謝に Glucuronic acid が如何なる影響を及ぼすかを検討し、大々興味ある実験成績を報じている。

他方 B₁ 投与による諸臓器の組織学的変化に関する報告は比較的少く、これは人体が B₁ の大量投与でも何ら副用を起すことなく経過する場合が多いためと思われる。然し先に渡理¹¹⁾は有精卵卵黄に B₁ 及び TPD の投与を行い鶏胎仔の肝臓に就き、又高柳¹²⁾は B₁ 及び TAD 投与の肝臓、腎臓及び心臓の組織学的変化に就いて大々報告している。

茲に於て余は当教室に於て行っている胎生期の薬理学的研究の一端として、更明確に B₁ 並びに TPD の作用を行い、孵化過程に於ける鶏胎仔の心臓及び腎臓に対する影響に就き組織学的に検索し、既往の業績と比較検討した。

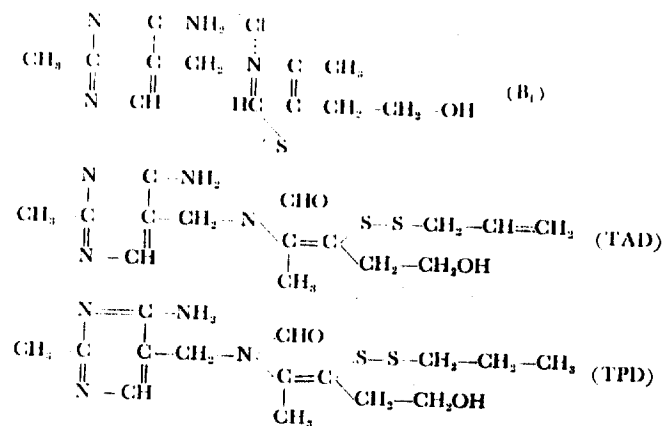
実験材料並びに方法

実験材料としては、50g 前後の新鮮なる白色レグホン種の鶏卵を使用し、無精卵及び不整卵等の異質卵を除外して、これを研究室に装置した自動調節器付電気式平面孵卵器¹³⁾に於て孵卵した。孵卵温度は、前半期は直上温を 101-102°F、後半期に於ては 102-103°F とした。湿度は 60-70% とした。孵化は林田⁷⁾の法に倣ひながら行った。経卵は 1 日朝夕 2 回、検卵は孵卵 5 日目に行い、無精卵及び死亡卵を除外し、孵卵 12 日目、15 日目

及び18日目の各卵に於て夫々死亡卵を除外して、任意に取出した5例宛の鶏卵子を実験に供した。

尚使用した薬物は B_1 塩酸塩及び TPD にて、これをアラビヤゴムに混じ、蒸留水にて乳剤とした。これは夫々の乳剤0.1cc中に1mg, 3mg, 5mg, 8mg, 10mg, 13mg, 15mg 及び18mg の8段階となる。鶏卵1個に0.1cc注射した。尚対照としてはアラビヤゴム乳剤 (Gummi arabicum 5mg/0.1cc) を鶏卵1個に0.1cc注射した。尚注射に際しては鶏卵の尖端部を消毒用アルコールにて清拭して、其の稍々外側には小孔を穿ち、ツバケウシ注射器及び1/2注射針を使用して、卵白内に所要の溶液を卵白及び葉液の流し込まない様に注意しつつ徐々に注入し、続いて小孔をワッフィンにて完全に封じ卵卵器に入卵した。本操作は無菌的に行つた。尚入卵個数は1段階100個宛とし、対照は80個を入卵した。標本作製に當つては、鶏心臓及び腎臓を摘出して型の如くホルマリン固定をなし、主としてこれをパラフィンにて包埋し、Hematoxylin-Eosin 複染色及び Azan-Mallory 染色を、更に一部に於て凍結切片を作製して Sudan III による脂質を行った。腎臓検査に當つて、原腎及び後腎の存在が認められるが余は後腎に就いて行つた。

B_1 及び TPD の構造式は次の通りである。



A. 心臓所見 (第1表及び第2表)

1. 孵卵12日目の場合

1) 対 照 例

心外膜に於ては多少の動脈硬化、肥厚はなく、一處に軽度の細胞浸潤があり、更に外膜の動脈起始部周囲の基底に於ける内皮細胞の増殖像が1例認められる。又心外膜血管に軽度の血液充満像がある。心筋層に於ては間質に細胞浸潤は全くなく、心筋自体に就いては、心筋線維は極めて細く、その間に於ける間隙が多少相違して居る像が認められる。その間隙中には数箇処であるが血液静止像がある。又心臓の大部分は動脈硬化を呈しない。心臓は其の大部分が動脈硬化を呈し、残つて見られる箇処に於ては動脈硬化は動脈硬化ではなく、心臓動脈硬化に於ても細胞浸潤及び水腫等は認められない。

2. B_1 注射例

1mg 注射例に於ては心外膜の動脈に軽度の細胞浸潤を認め、更に心外膜血管の血液充満が軽度にある。心筋層に於ては、心筋層の動脈血管に軽度の血液充満像がある。又心臓に於ては内皮細胞の増殖像が1例認められる。3mg 注射例に於ては心外膜に於ける細胞浸潤及び血液充満が1mg 注射例より軽度である。又心臓に於ては血液充満像が軽度にある。心臓に於ては内皮細胞の増殖像は前者より軽度である。心臓に於ては動脈硬化の増殖像が認められる。5mg 注射例に於ては心筋層の動脈血管に於ける血液充満像が軽度の細胞浸潤が軽度に見られる。又心臓に於ては動脈硬化の増殖像が1例認められる。8mg 注射例に於ては心外膜の細胞浸潤が軽度に見られ、心筋層に於ては動脈硬化の増殖像が1例認められる。10mg 注射例に於ては8mg 注射例と同様に、心筋層の血管充満が軽度に見られ、又

第 1 表

投与物質		対 照			V B ₁																							
解 卵		アラビアム			1 mg			3 mg			5 mg			8 mg			10mg			13mg			15mg			18mg		
学的所見		12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
心 臓	細胞浸潤	—	{	{	—	—	{	{	{	—	±	—	{	{	±	—	{	{	±	—	{	{	—	{	{	—	{	{
	血管充盈	{	{	—	±	+	{	{	{	—	—	{	—	—	{	±	—	—	{	±	—	{	±	—	{	{	—	{
	内臓の細胞浸潤	—	{	—	{	{	—	{	—	{	—	—	—	—	—	—	—	—	{	±	—	—	—	—	{	—	{	
心 筋	肥 大	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	脂肪類	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	自身壊死	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
血 管	細胞浸潤	—	±	—	—	—	{	—	{	{	—	{	—	—	{	±	—	{	±	—	{	±	—	{	±	—	{	±
	充 盈	{	±	±	{	±	—	±	±	{	±	{	±	±	{	±	±	{	±	—	±	±	±	{	±	±	±	
	出 血	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
心 内	結合組織増加	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	肥 大	—	{	—	{	—	±	—	—	—	{	—	{	{	{	—	—	—	{	—	—	—	—	—	±	{	—	
	増殖	—	{	—	{	—	—	—	—	—	{	—	{	{	—	—	—	—	{	—	—	—	—	—	—	—	—	—
腹 腔	水腫	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—
	細胞浸潤	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—	—

最も1例に極めて軽度で認められる。13mg 注射例では心外膜に細胞浸潤及び血管充盈が最大で認められ、又心臓の内臓細胞浸潤も軽度で認められる。心筋自身は1例に核の濃縮像が見られ、心筋層の血管及び心臓と肺との血管充盈があり、その周囲に細胞浸潤像が見られる。更に心内臓に於ても、内臓細胞の濃縮像があり、又1例に軽度の肥大像がある。尚内臓下組織に細胞浸潤のある例が1例で認められる。1mg 注射例では心臓の血管充盈及び細胞浸潤が軽度で認められ、又心筋層の血管充盈も見られる。18mg 注射例では心臓の血管充盈及び細胞浸潤が軽度で認められるのみで、その他の箇所には何等特記すべき変化は認められない。

3) TPD 注射例

1mg 注射例に於ては心外膜に軽度の血管充盈及び細胞浸潤を認め、又心臓の内臓細胞の濃縮像が見られる。心筋自身では1例に核の濃縮像があり、更に他の1例に細胞浸潤及び核の濃縮像が見られる。又心筋層の血管充盈が著明に認められる。3mg 注射例は心外膜に軽度の血管充盈及び細胞浸潤が認められる。心内臓には一部に軽度の内皮細胞の増殖が見られる。5mg 注射例は心外膜に軽度の血管充盈及び細胞浸潤が認められる。心内臓には一部に軽度の内皮細胞の増殖が見られる。8mg 注射例は心外膜に軽度の血管充盈及び細胞浸潤が認められる。心内臓には一部に軽度の内皮細胞の増殖が見られる。10mg 注射例は心外膜に軽度の血管充盈及び細胞浸潤が認められる。心内臓には一部に軽度の内皮細胞の増殖が見られる。13mg 注射例は心外膜に軽度の血管充盈及び細胞浸潤が認められる。心内臓には一部に軽度の内皮細胞の増殖が見られる。15mg 注射例は心外膜に軽度の血管充盈及び細胞浸潤が認められる。心内臓には一部に軽度の内皮細胞の増殖が見られる。18mg 注射例は心外膜に軽度の血管充盈及び細胞浸潤が認められる。心内臓には一部に軽度の内皮細胞の増殖が見られる。

投与物質

投与物質		対 照		V B ₁																									
				1 mg			3 mg			5 mg			8 mg			10mg			13mg			15mg			18mg				
腎 臓 組織学的所見	解 剖	ア ラ ビ ン	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
球 体	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
ホー マン 氏 袋	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
血 管	腎 臓	腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18
		腎 臓	12	15	18	12	15	18	12	15	18	12	15	18	12	15	18	12	15</										

浸潤を認め、心筋層の血管周囲にも同様に細胞浸潤が軽度に見られる。又心内膜に於ては内皮細胞の肥大像が認められる。8mg注射例では心外膜及び心筋層に夫々軽度の血管充溢を認め、更に心筋層の血管周囲には円形細胞の浸潤がある。10mg注射例では心筋層に軽度の血管充溢が見られるのみで、13mg注射例に於ては心外膜の細胞浸潤と心筋層の血管充溢が夫々軽度に認められる。15mg注射例では心筋の顆粒死及び末梢の循環障害像が一部に見られ、又心筋層に於ては血管充溢が、更にその周囲には細胞浸潤が軽度に認められる。18mg注射例では心外膜には細胞浸潤が軽度に、又心筋層に於ては末梢に循環障害像が認められ、更に血管充溢も著明に存在する。又結合組織の増殖も2例に於て夫々軽度に見られる。

I. 孵卵15日目の場合

1) 対 照 例

心外膜には細胞浸潤及び血管充溢を夫々軽度に認め、又間質にも細胞浸潤が軽度にある。心筋自身は12日目よりその線維が幾分太くなっているとはいえ、未だにその横紋模様は見られない。心筋層に於ては血管充溢が比較的著明であり、その周囲に全般的に細胞浸潤が軽度に見られる。心内膜では内膜下組織に変化はないが、内皮細胞の肥大及び増殖像が1例に於て著明に認められる。

2) B₁ 注 射 例

1mg注射例に於ては心外膜及び心筋層に血管充溢が見られ、又間質にも細胞浸潤が軽度にある。心外膜では内皮細胞の肥大及び増殖像が一部に認められる。3mg注射例では心外膜及び心筋層に円形細胞の浸潤及び血管充溢が夫々軽度に存在する。5mg注射例にも心外膜及び心筋層に軽度の血管充溢及び円形細胞の浸潤があり、又心筋層の一部に出血像の散見される例が見られる。更に心内膜に於て内皮細胞の肥大が軽度に見られる。8mg注射例では心外膜及び心筋層に細胞浸潤及び血管充溢が夫々軽度に見られ、又心内膜に於て内皮細胞の肥大が認められる。10mg注射例は心外膜に軽度の細胞浸潤を認め、心筋層では血管充溢及びその周囲の細胞浸潤が見られる。13mg注射例に於ては心外膜及び心筋層に夫々血管充溢及び細胞浸潤が認められ、心筋層は結合組織の増殖がある。又心内膜の内皮細胞に肥大及び増殖が軽度に見られる。15mg注射例の心外膜に軽度の細胞浸潤及び血管充溢が見られ、心筋層にも血管充溢がある。又心内膜に於て内皮細胞の肥大が軽度に見られる。18mg注射例では間質の細胞浸潤を軽度に認め、心筋層に於ては血管充溢がある。又その周囲に於て円形細胞の浸潤が夫々軽度に見られ、更に心内膜下組織に細胞浸潤が軽度にある。

3) TPD 注 射 例

1mg注射例は心外膜及び心筋層に血管充溢を軽度に認め、又間質及び心筋層に円形細胞の浸潤が夫々軽度に見られる。更に心内膜に於ては一部に内皮細胞の肥大像をみる。3mg注射例に於ては1mg注射例と多少の相違を認め、更に心筋層の血管周囲に細胞浸潤が見られる。又5mg注射例に於ても多少と1mg注射例と相違の変化が見られるのみである。8mg注射例には心外膜に著明な細胞浸潤と軽度の血管充溢がある。又心筋層にも軽度の細胞浸潤を見、心筋層に於ては一部に末梢の循環障害があり、更に心筋層の血管に軽度の血液充溢及び円形細胞の浸潤が認められる。10mg注射例は心外膜に円形細胞の浸潤と血管充溢を認め、更に心筋層の血管充溢も軽度に見られる。又心内膜には一部内皮細胞の肥大及び増殖が軽度に見られる。13mg注射例は心筋層の一部に末梢の顆粒死像が見られるのみである。15mg注射例では心筋層に血管充溢があり、又一部に末梢の循環障害及び心筋の顆粒死等が認められる。18mg注射例は心筋層の一部に限局性壊死像が見られ、又血管充溢像も著明に認められる。血管周囲に於て円形細胞の浸潤が軽度であり、結合組織の増殖も一部に見られる。心内膜の一部に内皮細胞の肥大を認める。

II. 孵卵18日目の場合

1) 対 照 例

心外膜には軽度の円形細胞浸潤を認めるが、血管充溢は見られない。心筋層の間質には細胞浸潤は全くなく、心筋自身も孵卵12日及び15日目より肥厚し、一部に於て横紋模様が見られる個例もある。尚心筋層には血管充溢が軽度にある。心内膜に於ては殆んど変化は認められない。

2) B₁ 注 射 例

1mg注射例には心外膜の肥厚した例があり、血管充溢及び間質の細胞浸潤も夫々軽度に見られる。3mg

注射例では僅かに心外膜の細胞浸潤と心筋層に於ける血管充溢が見られるのみである。5mg注射例に於ては、心筋層の一部に末梢の循環障害を認め、又心筋層には軽度の血管充溢も見られる。8mg注射例の心外膜には細胞浸潤と血管充溢があり、心筋層の一部に心筋の断裂像が見られる。又心筋層に血管充溢及び細胞浸潤が時々軽度に見られ、心内膜の一部に於て内膜下組織に水腫が認められる。10mg注射例の心外膜に細胞浸潤及び時々軽度に見られ、又心筋層の細胞浸潤も軽度に見られる。心筋層に於ては一部に末梢の循環障害及び心筋の断裂像が見られる。13mg注射例では心筋層の血管充溢が軽度に見られるのみである。15mg注射例の心外膜に細胞浸潤を認め、更に心外膜及び心筋層に血管充溢が時々軽度に見られる。又心内膜に於ては時々軽度の水腫が認められる。18mg注射例では心外膜に血管充溢があるのみで他に特記すべき変化は見られない。

3) TPD 注射例

1mg 注射例は心外膜に血管充盈があり、間質には細胞浸潤が見られる。尚心筋層の一部に末梢の循環障害が認められる。3mg 注射例の一部に心外膜の肥厚が見られる。又間質の細胞浸潤及び心内膜の内皮細胞の増殖が少々軽度にある。5mg 注射例に於ては心外膜の一部に肥厚像が見られ、又外膜に細胞浸潤も存在する。心筋層には末梢の循環障害を認め、血管の充盈も見られる。8mg 注射例では心外膜及び心筋層に血管充盈があり、又細胞浸潤を心外膜及び心筋層血管周囲に認める。尚間質にも同様に細胞浸潤がある。更に心筋層の一部に末梢の循環障害があり、心筋自身に於ても一部に洞洞腫脹が見られる。10mg 注射例の心筋層に於ては軽度の細胞浸潤を見、又血管には血液充盈像があり、更にその周囲には細胞浸潤が認められる。尚心筋層の一部に末梢の循環障害があり、心内膜には内皮細胞の増殖が一部に軽度に見られる。13mg 注射例の心外膜に軽度の血管充盈を認め、心筋層に於ては間質の細胞浸潤及び末梢の循環障害像が存在している。心筋自身にも軽度の細胞浸潤が見られ、心筋層の血管にも血液充盈像があり、血管周囲には円形細胞の浸潤がある。心内膜には内皮細胞の肥大像が認められる。15mg 注射例では心外膜に細胞浸潤を軽度に見、一部の心筋層には洞洞腫脹死の变化が局限して処々に認められ、更に心筋層に血管充盈及び血管周囲の細胞浸潤が存在している。心筋に於ても内皮細胞の肥大及び増殖像が認められる。18mg 注射例に於ては心外膜に血管充盈があり、又間質の細胞浸潤も見られる。心筋層には処々に軽度の限局性の壊死像が認められ、更に血管の充盈及び血管周囲に軽度の細胞浸潤が見られる。心内膜では内膜下組織に軽度の細胞浸潤があり、内皮細胞の肥大及び増殖も少々軽度にある。

B. 腎臟所見 (第3及び第4表)

1. 舞卯12日目の場合

1. 对照例

糸球体は、その組織として、均一でない。糸球体は大小不同にして、ボーマン氏嚢と糸球体の癒着はない。その外には、しばしば核が見られる。間質には血管の充満血があり、その周囲に細胞膜調が軽度に変化する。糸球体嚢は全く正常である。

2. 注射例

1mg 注射時には、Aorta 及びその分枝に種大動脈が見られ、細尿管の一部に硝子様肉柱が軽度に見られる。腎臓には、Aorta 及びその分枝の細動脈流が軽度に見られる。3mg 注射例では糸球体は全例で正常で、Aorta 及びその分枝の動脈流は軽度に見られる。細尿管には硝子様肉柱が一部に存在する。腎臓は軽度動脈硬化を呈し、Aorta 及びその分枝の動脈流は軽度に見られる。5mg 注射例は細尿管に顆粒状変化及び硝子様肉柱が一部に存在する。腎臓は軽度動脈硬化を呈し、Aorta 及びその分枝の動脈流は軽度に見られる。8mg 注射例に於ても硝子様肉柱及び顆粒状変化は軽度に見られる。Aorta 及びその分枝の動脈流は軽度に見られる。10mg 注射例では糸球体は全例で正常である。ボーデン氏嚢との癒着像も幾々に見られる。Aorta 及びその分枝の動脈流は軽度に見られる。13mg 注射例は細尿管に顆粒状変化及び硝子様肉柱が一部に存在する。腎臓は軽度動脈硬化を呈し、Aorta 及びその分枝の動脈流は軽度に見られる。15mg 注射例では顆粒状変化及び硝子様肉柱が一部に存在する。腎臓は軽度動脈硬化を呈し、Aorta 及びその分枝の動脈流は軽度に見られる。18mg 注射例では糸球体は軽度大し、充血像を呈している。細尿管は軽度動脈硬化を呈し、Aorta 及びその分枝の動脈流は軽度に見られる。又軽度の顆粒状変化があり、更に間質血管の腫脹も軽度に見られる。

第 3 表

投与物質		T P D											
		1 mg			3 mg			5 mg			8 mg		
		12	15	18	12	15	18	12	15	18	12	15	18
心臓組織学的所見		12	15	18	12	15	18	12	15	18	12	15	18
心外膜	細胞浸潤	+	+	-	+	+	+	+	+	+	+	+	+
	血管充満	+	+	+	+	+	+	+	+	+	+	+	+
	肉質の細胞浸潤	+	+	-	+	+	+	+	+	+	+	+	+
心筋	肥 大	-	-	-	-	-	-	-	-	-	-	-	-
	萎 縮	-	-	-	-	-	-	-	-	-	-	-	-
	筋 断 裂 死	-	-	-	-	-	-	-	-	-	-	-	-
心 血 管	細胞浸潤	-	-	+	-	-	+	-	-	+	-	-	+
	充 満	+	+	+	+	+	+	+	+	+	+	+	+
	結合組織増加	-	-	-	-	-	-	-	-	-	-	-	-
心 内 膜	肥 大	-	-	+	-	-	+	-	-	+	-	-	+
	増 殖	-	-	+	-	-	+	-	-	+	-	-	+
	内 水 腫	-	-	-	-	-	-	-	-	-	-	-	-
心 下 組 織	細胞浸潤	-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-
		-	-	-	-	-	-	-	-	-	-	-	-

3) TPD 注 射 例

1 mg 注射例ではボーマン氏囊と糸球体の癒着している例があり、細胞質には一部に硝子様円柱が見られる。血管の鬱血も軽度認められる。3 mg 注射例に於ても1 mg注射例と同様に糸球体は癒着した例が一部に見られ、一部糸球体は内腔の癒着が存在し、更に一部に硝子様円柱及び顆粒状物が認められる。5 mg 注射例では糸球体の腫大像を一部に見、又一部は癒着した例が一部に見られる。6 mg 注射例には顆粒変性及び硝子様円柱が大部分に認められ、間質血管の鬱血も認められる。8 mg 注射例では糸球体の充血があり、細胞質には顆粒変性を処々に於て軽度に見え、間質血管には鬱血が認められる。10 mg 注射例にも糸球体の腫大を呈している例があり、又その充血も一部に見られる。細胞質には顆粒変性が軽度に見え、間質血管の鬱血も認められる。13 mg 注射例では糸球体の腫大が軽度に見え、細胞質には顆粒変性が一部に見られる。15 mg 注射例では糸球体の腫大が軽度に見られる。15 mg 注射例では糸球体の腫大が軽度に見え、細胞質には顆粒変性が一部に見られる。18 mg 注射例は処々の糸球体が腫大し、又糸球体の前葉も一部に認められる。

第 4 表

[illegible]

マン氏囊の拡張を起している例がある。細尿管に於ては軽度の内腔の拡張と、一部に顆粒変性及び硝子様門柱が夫々見られる。

1) 孵卵15日目の場合

対 照 例

糸球体は12日目比し、全般に縮小しており、ボーマン氏囊と癒着している例もある。細尿管は全例に於て軽度であるが核の消失像が認められる。間質血管に於ては充鬱血像が著明に見られる。

2) B₁ 注 射 例

1mg注射例では細尿管に軽度の核の消失を認めるのみであるが、3mg注射例に於ては細尿管の核の消失と変性が夫々軽度に見られる。5mg注射例の細尿管に顆粒変性及び核の消失を夫々軽度に見え、一部に硝子様門柱があり、又内腔の狭小像も処々に見られる。8mg注射例では糸球体の縮小が全射に見られ、細尿管に於て内腔の狭小が一部にあり、又軽度の類壊死をも伴っている。10mg注射例では糸球体の縮小が処々にあり、管には軽度の顆粒変性及び空胞化が見られ、一部に於て核の消失がある。13mg注射例は10mg注射例と同様糸球体が全般に小である。細尿管では内腔の狭小が軽度であり、又一部に於て顆粒変性が認められ、間質血管に充鬱血がある。15mg注射例では糸球体の腫大が一部に見られ、間質血管の充鬱血が軽度に存在するのみで、18mg注射例では糸球体の一部に萎縮が見られ、又ボーマン氏囊と糸球体との癒着した像が一部に認められ、更に間質に於ては結合組織の増殖と、血管の充鬱血が軽度に見られる。

3) TPD 注 射 例

1mg注射例に於ては一部の糸球体に腫大像を見、糸球体とボーマン氏囊との癒着も一部に認められる。細尿管は内腔の狭小像が処々に見られ、顆粒変性及び硝子様門柱も夫々軽度にある。又間質血管の充鬱血を伴っている。3mg注射例では糸球体の腫大している例があり、細尿管の顆粒変性及び核の消失を認め、一部に限局し類壊死～類壊死の像がある。間質血管の充鬱血も軽度に見られる。5mg注射例に於ては一部にボーマン氏囊と糸球体との癒着を見、細尿管には3mg注射例より著明な顆粒変性及び核の消失像を認め、更に類壊死の像が一部に存在する。間質には充鬱血があり、その周囲に軽度の細胞浸潤がある。8mg注射例の糸球体は大小不同で、系の崩壊が一部に認められ、間質には血管充鬱血が認められる。10mg注射例では一部の糸球体に萎縮像が見れる。尚細尿管及び間質では殆んど変化は認められない。13mg注射例に於ては一部の糸球体に萎縮像を見、細尿管でも顆粒変性で内腔の狭小像が認められる。15mg注射例では一部の細尿管に顆粒変性と硝子様門柱が認められ、間質血管の充鬱血も軽度に存在する。18mg注射例では糸球体の縮小が一部にあり、細尿管に於ては顆粒変性、核の消失及び硝子様門柱等が見られ、一部に極く軽度の類壊死がある。間質血管には充鬱血を軽度に認める。

II. 孵卵18日目の場合

1) 対 照 例

糸球体は大小不齊はなく、殆んど同一の大きさを示しており、ボーマン氏囊は殆んど全例に於て正常である。細尿管には一部に顆粒変性及び空胞化が軽度に見られ、核の消失も一部に認められる。硝子様門柱は認められていない。間質には変化はない。

2) B₁ 注 射 例

1mg注射例に於ては一部に糸球体の崩壊像を認め、細尿管では著明な顆粒変性及び核の消失像が見られる。又一部の細尿管に類壊死が認められる。間質では脂肪染色により処々に小脂肪滴が存在している。間質血管には軽度の充鬱血がある。3mg注射例では1mg注射例と同様に細尿管腔の狭小を一部に認め、顆粒変性、核の消失及び脂肪変性等を夫々軽度に見、又一部に於ては類壊死の像を認める。間質には充鬱血が軽度にある。5mg注射例では糸球体の一部に充血を見、細尿管には顆粒変性、核の消失及び硝子様門柱等を軽度に見、又類壊死も見られる。間質には円形細胞の浸潤及び血管の充鬱血を軽度に認め、その血管周囲に於ては細胞浸潤がある。8mg注射例は糸球体の萎縮が処々に著明に認められる。細尿管腔は僅かに狭小となり、顆粒変性及び核の消失が軽度であり、更に一部に類壊死が見られる。尚間質血管には充鬱血が軽度に認められる。10mg注射例に於ては細尿管には顆粒変性、核の消失及び脂肪変性等が夫々軽度であり、更に一部に類壊死像も見られる。間質には明

脂肪浸潤と血管の充塞血及びその周囲に円形細胞の浸潤がある。13mg 注射例に於ては細尿管に腔の狭小があり、顆粒変性、核の消失及び硝子様円柱等を軽度に見、又類壊死を全般に認める。間質には細胞浸潤及び充塞血が軽度にある。15mg 注射例では糸球体の萎縮を全般に認める。細尿管には顆粒変性及び核の消失を、又空胞化及び脂肪変性をも軽度に見、更に一部に於て類壊死を認める。又細尿管腔の狭小も認められる。18mg 注射例に於ては糸球体の萎縮を軽度に見、ボーマン氏嚢の一部に癒着がある。細尿管には著明な顆粒変性があり、核の消失及び空胞化も認められ、全般に類壊死～壊死の像がある。又細尿管腔の狭小も一部に認められる。間質血管の充塞血が軽度にある。

3) TPD 注射の場合

1mg 注射例では細尿管に軽度の顆粒変性、核の消失及び脂肪変性等があり、一部に於ては類壊死がある。又細尿管腔の狭小も軽度に見られる。3mg 注射例では糸球体の萎縮が軽度に見られ、細尿管に於ては顆粒変性及び核の消失を軽度に見、脂肪変性及び類壊死も一部に夫々認められる。又間質血管の充塞血も軽度に見られる。5mg 注射例は糸球体に軽度の萎縮があり、細尿管には顆粒変性及び脂肪変性が見られる。間質血管の充塞血も軽度にある。8mg 注射例では糸球体の一部に萎縮を認め、細尿管の顆粒変性、脂肪変性及び類壊死、進行変性が夫々軽度にある。間質に於ては円形細胞の浸潤及び充塞血がある。10mg 注射例は細尿管の一部に顆粒変性、脂肪変性及び核の消失等の変化が夫々軽度に見られる。又間質血管の充塞血も著明である。13mg 注射例では一部にボーマン氏嚢と糸球体の癒着を認め、細尿管には顆粒変性及び脂肪変性が夫々軽度に見られ、間質には充塞血がある。15mg 注射例では糸球体の一部に萎縮があり、細尿管には顆粒変性、核の消失及び空胞化が夫々軽度に見られ、又間質には著明な充塞血がある。18mg 注射例では糸球体の一部に筋系の崩壊像を認め、細尿管に於ては顆粒変性、核の消失及び硝子様円柱等が見られる。間質血管には15mg 注射例と同様に著明な充塞血像がある。

総 括

余は鶏卵に B_1 及び TPD を注射し、孵卵経過中の12日目、15日目、及び18日目に於て鶏胎仔心臓及び腎臓に就て組織学的に検索を試みた。

これに先立ち対照例のアラビヤゴム注射例について検討したが、心臓所見は心外膜及び心筋層に血管充塞を見、その他12日目に外膜及び間質の細胞浸潤を軽度に見るのみで特に変化はなく、高橋のアラビヤゴム注射例或は岡田¹⁰⁾の報告した無処置或はリニゲル注射例と比較するに、概ね同様の成績である。又腎臓に於ては糸球体が初期(12日目)に幾分腫大して見られるが15日目には逆に縮小され、細尿管には核の消失が認められる。18日目には細尿管に顆粒変性及び空胞化を伴っている。これらの所見は岡田の行った無処置及びリニゲル氏液注射の報告には記載を見ない。又和田の報告によれば対照例では18日目に於て細尿管細胞が内皮層に接する部位、増殖中に空胞様欠損部が認められるとしているが顆粒変性は見られていない。尚高橋のアラビヤゴム注射例では12日目、15日目及び18日目に於て夫々細尿管の顆粒変性を認め、更に15日目には核消失を伴っている。即ち余のみに見られた細尿管の顆粒変性は高橋の成績と類似しており、更に鈴木¹¹⁾の報じた腎臓の死後変化と一致している。又腎臓に於ては尿細管細胞は核の変化を伴わず、原形質は一般に膨化して、微細顆粒の発現する変化であることに就いては古木¹²⁾を参照しているが、その本質に就いては未だ明らかにされない。本報告は尿細管細胞の変化が死後の変化によって引き起こされた場合、多くは核の変質が認められるとし、腎臓細尿管上皮部の細胞増殖が減少して、尿細管が萎縮し、尿細管腔の全般的な閉塞が起る場合がある。尿細管細胞は尿細管腔の閉塞を起すものではないと報告している。

B_1 注射例に就いて総括すると、心臓所見では各濃度注射例に心外膜及び心筋層の血管充塞、その他心臓の細胞浸潤が心外膜、間質及び血管周囲等に見られている。又5mg及び10mg注射例の孵卵18日目に心臓の中心に未熟の筋線隙を認めるのみであり、その他特記所見はない。尚高橋は B_1 の1mg、5mg、10mg及び15mgの4濃度に就いて実験を行い、 B_1 注射の心臓所見として孵卵初期に於て心外膜の肥厚を、又低濃度注射例では孵卵中期に、高濃度注射例では孵卵後期に間質の細胞浸潤を認め、更に孵卵後期に於て結合織の増殖を認めて

るが、これ等の変化は夫々軽度であり、余の場合と大差ない成績である。西沢は解卵初期に於ける B₁ の測定した処、心臓、肝臓及び小腸では初めは僅少であるが、日を経るに従って臓器の发育と共に増量すると報じ、又新生児では成人と同様に各臓器 B₁ 濃度には順序があり、心臓が最大で、肝、腎がこれに次ぐと井田等は記載をしている。斯くの如く B₁ は心臓に著明な親和性がある。尚組織学的変化に就いて白石等は幼若白鼠に B₁ 塩酸塩の 0.5mg を 60 日間連続皮下注射し、心臓には対照例と何等異なる所見はないと報じているが、余の実験の胎生期動物に於ても大体同様の傾向である。次に腎臓所見は対照に比し、糸球体及びボーマン氏囊に於ては全變に殆んど変化はないが、細尿管には顆粒変性が解卵後期に各濃度の注射例共著明となつてきている。又硝子様円柱も 5mg 注射例に於て各実験破産日を通じて軽度に見られており、又その他の濃度の注射例にも認められる。尚低濃度注射例は解卵 18 日目に、高濃度注射例は解卵初期より、細尿管の顆粒変性或は顆粒死乃至壊死等の進行変性を呈している。これ等の所見は毒物による一種のネフローゼ様の変性と考えられる。腎臓の上記の成績は白石の報告とは反するが、これは胎生期の場合と成長動物の B₁ の組織に対する感受性の差異と思われる。高橋は B₁ 注射例の解卵初期では殆んど変化は見ないが、解卵後期に於ては糸球体に円形細胞の浸潤並びに細尿管の顆粒死乃至壊死を認めており、細尿管の変化は余の成績と類似している。又高橋等はネーデルの場合 B₁ 投与量を増めると諸臓器の B₁ 濃度が高くなるが、飽和に達した時の高い B₁ 濃度を示す臓器は肝、心、腎等で 1000 μ g/g 又はそれ以上に達すると報告しており、B₁ 投与の場合心臓及び肝臓に次いで腎臓にもかなりの含有量を認めていることは周知の事実である。

TPD 注射例の心臓所見は心外膜の細胞浸潤及び血管充盈或は心筋内の血管充盈及び血管周囲の細胞浸潤等で概ね実験各期を通じて見られ、又心内膜には内皮細胞の肥大が高濃度注射例の解卵後期に於て認められている。その他心筋内には低濃度注射例では末梢血管の循環障害があり、又高濃度注射例では解卵各期に於て軽度であるが限局性の顆粒死乃至壊死が見られている。この顆粒死乃至壊死は B₁ 注射例には全く見られなかった変化であり、TPD は B₁ より心臓に対しては毒性が強いのではないかとと思われる。又高橋の TAD 注射の場合解卵初期に於ては心外膜の細胞浸潤並びに可逆性と思われる空胞形成が見られ、10mg 注射例の解卵 18 日目の一部に顆粒死乃至壊死を呈しているのが認められるが、氏の場合に於ても B₁ 注射例には全く見られないこと等からして TAD と TPD の心臓に及ぼす影響は似ているものと考えられる。尚渡辺の報じた鶏卵白肝臓の場合に於ても TPD 注射の場合、高濃度注射例の解卵後期に顆粒死乃至壊死が見られ、同様に林田の実験でも高濃度の場合 TPD 注射例が B₁ 注射例に比し強い发育障害をみており、余の成績と対比すると发育障害、肝臓障害及び心臓障害の傾向は高濃度注射例に於ては概ね一致している。松川、鈴置等¹⁰⁾は家兎の B₁ 及び TPD 両投与群に於て臓器 B₁ 値及び附屬率を検し、肝、腎、脳では大差なく、心臓の B₁ 値が TPD 投与群で最も増大することを認め、TPD が少なくとも心臓に於ては B₁ より親和性の強いことが容易に納得出来る。このことは余の成績と何等かの関係があるのではないかとと思われる。

腎臓所見に於ては糸球体及びボーマン氏囊は B₁ 注射例と同様で殆んど変化は見られない。細尿管では顆粒変性が概ね各濃度の注射例に認められ、硝子様円柱も B₁ 注射例と同程度に見られているが、細尿管の顆粒死は 1mg、3mg、8mg、及び 13mg 注射例の解卵 18 日目にのみ軽度認められている。即ち余の腎臓に於ては TPD 注射例と B₁ 注射例を比較すると組織障害は TPD 注射例は軽度である。尚高橋の TAD 注射例では解卵後期に於て 1mg、5mg、10mg 及び 15mg の各濃度に細尿管の著明な顆粒変性並びに核消失を伴う顆粒死乃至壊死が認められ、この所見により TAD は TPD より細尿管の病的変化を強度に來たと述べても過言ではなからうと思われる。

以上要するに、B₁ 及び TPD 注射に於ける心臓及び腎臓の組織学的変化は總体的にみて、夫々比較的軽度であり、一部の濃度に見られた進行変性も可逆性のものと思われる。

結 論

B₁ 並びに TPD を夫々 8 濃度に分け、有精鶏卵に注射し、解卵 12 日目、15 日目及び 18 日目に各臓器の B₁ 濃度及び腎臓に於て組織学的検査を行い、次の結論を得た。

1. B₁注射例では心臓に於て殆ど変化は見られないが、腎臓では低濃度注射例は腎臓後期には、高濃度注射例は腎臓初期より細尿管の可逆性と思われる顆粒変性並びに核消失を伴う顆粒死乃至壊死等の変化が認められる。

2. TPD注射例では心臓に於て低濃度注射例では間質の細胞浸潤を認め、高濃度注射例では腎臓初期に顆粒の顆粒死乃至壊死の変化が見られ、更に内皮細胞の肥大及び増殖が認められる。腎臓に於ては腎臓初期で高濃度に細尿管の顆粒変性が見られ、腎臓後期では低濃度注射例の細尿管に可逆性の顆粒変性並びに核消失、顆粒死乃至壊死等の変化が認められる。

稿を終るに臨み終始御懇篤なる御指導並びに御校閲を賜わった恩師角尾教授に対して衷心より感謝の意を表します。助を賜わった山口、川北両博士に対し深く感謝の意を表します。

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附 録 図 説 明

胎仔心臓標本 (Haematoxylin Eosin 複染色)

- 第1図. 胎期12日 B₁ 3mg 注射例
- 第2図. 胎期12日 TPD 1mg 注射例
- 第3図. 胎期15日 B₁ 3mg 注射例
- 第4図. 胎期15日 B₁ 15mg 注射例
- 第5図. 胎期18日 B₁ 18mg 注射例
- 第6図. 胎期18日 B₁ 18mg 注射例
- 第7図. 胎期18日 TPD 15mg 注射例
- 第8図. 胎期18日 TPD 18mg 注射例

胎仔腎臓標本 (Haematoxylin Eosin 複染色)

- 第9図. 胎期12日 B₁ 15mg 注射例
- 第10図. 胎期12日 TPD 18mg 注射例
- 第11図. 胎期15日 B₁ 8mg 注射例
- 第12図. 胎期15日 TPD 1mg 注射例
- 第13図. 胎期15日 TPD 13mg 注射例
- 第14図. 胎期18日 B₁ 15mg 注射例
- 第15図. 胎期18日 TPD 15mg 注射例
- 第16図. 胎期18日 TPD 18mg 注射例

IMPROVEMENT OF COLOR VISION BY VITAMIN INTAKE

By Donald P. LeGalley, Ph. D.,* and J. W. E. Harrison,
P. D., Ph. M.**

A SERIES of experiments have been performed to test whether or not large dosages of Vitamin A, Vitamin B₁, (Thiamine Hydrochloride) or Vitamin B₂ (Riboflavin) produce improvements in the color vision of color blind subjects.

When it is realized that approximately 4% of the men applying to the armed services are turned down because of defective color vision, then the desirability of finding a method of improving this defect is realized. Of course on the average this same percentage of rejections applies to all industries where color blindness or lack of clear color vision is a liability, such as airplane pilots, railroad employees, and chemists.

Our attention was called to this problem, when quite a number of our students who were trying to join the various armed reserve forces were turned down because of defective color vision. We were interested first in testing these students to see just how badly color blind they were, and second to see if this defect could be improved by any dietary procedure such as an increased vitamin intake.

The total number of color blind subjects available were divided up into four groups of four each, so that each group contained on the average about the same degree of color blindness. During the experiment some groups were reduced to three because the subjects were called by the selective service, joined the armed forces, or, as in one case, left school for other reasons.

One group of four was put as a control, i. e. they were not given additional vitamins, but tests were made on them once a week at the

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same time, and in the same way that tests were made on the rest of the groups. To another group of four was administered 30,000 U. S. P. units of Vitamin A daily. Since the normal minimum adult need of Vitamin A is 5,000 U. S. P. units, they received a dosage about six times normal in addition to that obtained in their regular diet. To the members of another group of four was given 8 mg. of Vitamin B₁ daily. Since the normal minimum daily need of this vitamin is about 1 mg., their daily dosage was about eight times normal. The fourth group of four received 16 mg. of Vitamin B₂ daily. The normal minimum daily dosage of this vitamin is about 2 mg., so these subjects also had a dosage eight times normal.

No attempt was made to control the daily character of the food and it may be presumed that all were on an average diet.

The color vision of each subject was tested carefully at the beginning before any vitamins were administered, and each week thereafter for a period of ten weeks. The tests were made with the aid of color blind charts secured from the American Optical Co. These charts are a combination of the best Ishihara and Stilling charts, along with some American charts, and are made up of colored dots arranged so as to form certain numbers, letters, traces, or blanks.

The color blind sees different numbers, letters, or traces, than the normal person, or none at all, and therefore his color vision can be measured without naming the colors.

There is a total of eighty-five possible answers, and a careful record was kept of the number of right and wrong answers given by each subject at each reading. The number of wrong answers divided by eighty-five was taken as the per cent. of color vision deficiency. The charts were cut apart and shuffled like a deck of cards each week, so that the subject had no chance of memorizing or learning them in sequence. The operator was very careful not to indicate to the subject whether the answers given were correct; he merely recorded the numbers, etc., which the subject read.

Illumination was furnished by a large north light window which covered one side of the room. No artificial illumination was used. No readings were taken on extremely dark days, although when this point was tested it was found to have very little influence on the reading of the color charts. All subjects made their readings on the same afternoon, and therefore the illumination was about equal for all. In addition the intensity was measured with a Weston exposure meter

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and was found not to vary appreciably from week to week. To check this point further, two non-color-blind subjects were asked to read the charts each week along with the rest of the group. Of the eighty-five possible answers, one subject never gave a single wrong answer, while the other one averaged two errors per week, but these did not depend on whether the day was bright or dark.

It is recognized these charts are probably not as accurate a method of measuring color blindness as the skein matching procedure, or the intensity measuring method with the spectroscope used by the Bureaus of Standards. But since the chart method is so convenient, and since the patient can be tested in five minutes by this method, they were used. The little which is lost in accuracy is made up for by the time one saves, and the ease with which the determinations can be made.

It is also recognized that several workers, among them Dunlap and Loken (1), have recorded in the scientific literature improvements of color blindness as a result of administering large dosages of Vitamin A. However, it appeared desirable to check these results and in addition to make studies with Vitamin B₁ and Vitamin B₂. It is also recognized that groups of four represent a small sampling, but it is believed that since the results within a group checked one another, that the average result is fairly reliable.

One of the purposes of running a control group was to measure the amount of "learning," due to the fact that the subjects were reading the same charts each week. The tests showed that the average improvement of this group over a period of ten weeks was only 2.2%, which indicated that "learning" was not appreciable. Additional tests indicated that the charts were a true test of color vision. Besides shuffling them thoroughly between the readings each week, sometimes repeat readings were taken at random. Also if either "Learning" or "Memory" were factors, there would have been a rise in the number of wrong answers taken immediately after the two-week Christmas holiday recess, since there would have been some "forgetting" during this period.

RESULTS OF TEST

Vitamin Group	Normal Min. Daily Need	Dosage Administered	Average Improvement	Additional Improvement After Switching
Control		None	2.2%	X X X
A	5,000 U. S. P. units	30,000 U. S. P. units	20.7%	A to B ₁ 11.8%
B ₁	1 Mg.	8 Mg.	22.3%	B ₁ to A 10.8%
B ₂	2 Mg.	16 Mg.	2.5%	X X X

The results of the tests are shown in the table. Essentially the points are: whereas the control group showed an improvement of only 2.2%, those to whom 30,000 U. S. P. units of Vitamin A were administered showed an average improvement of 20.7%, and those to whom 8 mg. of Vitamin B₁ was administered showed an average improvement of 22.3% over the ten-week period. On the other hand, those to whom the 16 mg. of Vitamin B₂ was administered showed an improvement of only 2.5% over the same period. It might be concluded that since the 20.7% improvement for Vitamin A subjects, and the 22.3% improvement for the Vitamin B₁ subjects is so much greater than the 2.2% improvement of the controls that concentrated dosages of either of these two vitamins is effective in producing improvement in color vision. As mentioned, results have been published for Vitamin A, but it is believed that this is the first time that Vitamin B₁ has also been found to be effective. On the other hand, large dosages of Vitamin B₂ were found to produce practically no improvement in color vision. It is of interest to note that most of the improvement in the case of the Vitamin A subjects occurred in the first five weeks, after which the readings reached a plateau. The Vitamin B₁ subjects took longer to reach this plateau, but in both cases with Vitamin A and B₁ nearly all of the improvement possible was secured within the ten-week period.

After the plateau was reached with one vitamin, tests were made to note if the addition of another vitamin would produce further improvement. In the case of those to whom the 30,000 U. S. P. units of Vitamin A had been originally administered, and who had reached the plateau of improvement, 8 mg. of Vitamin B₁ per day was administered for another period of ten weeks. In this case an additional improvement of 11.8% was noted. In the case of those who had received the Vitamin B₁ during the first ten-week period, 30,000 U. S. P. units per day of Vitamin A was administered. This

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group showed an additional improvement which averaged 10.8%. Since in both cases the additional improvements were produced after they had reached a plateau with the first vitamin it can be concluded that both Vitamin A and Vitamin B₁ are effective in producing improvement in color vision even under adverse conditions. When further improvement was tried by administering 16 mg. of Vitamin B₂ over a second ten-week period, it was found that when it followed Vitamin A the improvement averaged 2.7%, while when it followed Vitamin B₁ the average improvement was 3.5%. In both of these cases the improvement produced by additional Vitamin B₂ can be considered negligible.

The vitamins for this experiment were kindly furnished by Hoffmann LaRoche, Inc. of Nutley, N. J., and the authors are indebted to them for this supply. They were encapsuled under the direction of Mr. Loy Packer, a senior pharmacy student. The authors also wish to express their appreciation to the students and faculty members who cooperated in this program, and to Dr. H. C. Wood, Jr., Professor of Pharmacology at the Philadelphia College of Pharmacy and Science, and to Dr. Julius Neumaeller of the Pennsylvania State College of Optometry, for suggestions and advice.

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A STUDY OF THE TOXICITY OF CERTAIN VITAMIN PREPARATIONS IN AN EXPERIMENT

G. N. Lipkan, Kiev

Vitamins are a group of compounds which have been widely studied. However in the literature available to us we encountered only isolated works touching on the toxicity of these biologically active substances (A. M. Timofeyeva and G. A. Tsofina, 1943; Ya. B. Maksimovich, 1963; Yu. M. Ostrovskiy *et al.*, 1968; Winter *et al.*, 1967).

We studied the toxicity, in acute experiments, of several water-soluble vitamins of the B group, ascorbic acid, a preparation of vitamin P - rutin, and palascorbine, which possesses C and P vitamin activity. The experiments were conducted on white rats which had been placed on the usual diet. Toxicity was determined using a probe-analysis method proposed by Litchfield and Wilcoxon (M. L. Belen'kiy, 1959). In all the experiments, the vitamin dosage was determined which caused the death of all the experimental animals, as well as the size of the dose which caused no deaths. Five animals were used for testing each dose. Solutions of the preparations were made immediately before administration in distilled water. The incompletely soluble substances were administered in the form of a suspension. Death of the animals was calculated over 2-3 days. The data obtained are reflected in Tables 1 and 2.

Of all the preparations we studied, vitamin B₁ and folic acid had a relatively high toxicity with intraperitoneal administration. The phosphorylated form of thiamine, co-carboxylase, under the experimental conditions, was approximately 5 times less toxic than vitamin B₁ itself.

In clinical practice, the pharmacodynamic effect of vitamin preparations is even more widely utilized. However increased doses of vitamins are often used without sufficient experimental grounds resulting from empirical observations and conclusions, while only isolated attempts in some way or other occur on which to base the possible application of increased doses of the vitamins with the aim of therapeutics. Z. T. Malkin (1952) concluded that in order to

Table 1. Acute Toxicity of Vitamin Preparations with a Single Intraperitoneal Administration to Rats (mg/kg)

Preparation	Max. Tolerab. Dose (LD ₀)	LD ₁₆	LD ₅₀ & its Reliab. Lim. for p = 0.05	LD ₈₄	Abs. Leth. Dose (LD ₁₀₀)
Vitamin B ₁ (thiamine)	300	335	390 (342-445)	475	500
Co-carboxylase	1600	1655	1955 (1746-2190)	2300	2300
Vitamin B ₂ (riboflavin)	3000	-	4000	-	-
Nicotinic acid	700	820	1080 (900-1296)	1400	1500
Vitamin B ₆ (pyridoxine)	1600	1675	1925 (1750-2118)	2195	2200
Folic acid	500	610	720 (600-864)	850	900
Orotic acid	1500	1875	2200 (1880-2574)	2810	3000

Table 1 concluded

Ascorbic acid	2100	2200	2499 (2251-2774)	2810	3000
Rutin	15,000	-	20,000	-	-
Galascorbine	2800	2850	3120 (2943-3307)	3450	3500

obtain the pharmacodynamic effect of the vitamin preparations, it is necessary to use doses 10-20 times greater than the physiological doses, which reflect man's requirements for these vitamins. If you start with the physiological norms of man's daily requirement of these vitamins, established in 1960 by the Ministry of Health Protection in the USSR (N. S. Yarusov, 1961) and with the calculation principle presented above for doses of vitamin preparations having a pharmacodynamic effect, then for a man weighing 70 kg on the average, the curative doses are within the limits of 20-60 mg for vitamin B₁, 25-70 mg for vitamin B₂, 1000-2400 mg for vitamin C, 150-500 mg for nicotinic acid, and 20-40 mg for vitamin B₆. According to P. I.

Table 2. Acute Toxicity of Vitamin Preparations with a Single Subcutaneous Administration to Rats (mg/kg)

Preparation	Max. Tolerab. Dose (LD ₀)	LD ₁₆	LD ₅₀ & its Reliab. Lim. for p = 0.05	LD ₈₄	Abs. Leth. Dose (LD ₁₀₀)
Vitamin B ₁ (thiamine)	700	760	840 (785-899)	930	1000
Co-carboxylase	4000	4250	4650 (4346-4976)	5170	5200
Vitamin B ₂ (riboflavin)	5000	-	8000	-	-
Nicotinic acid	2000	2050	2550 (2179-2984)	3125	3200
Vitamin B ₆ (pyridoxine)	2600	2750	3130 (2653-3693)	3600	3800
Folic acid	3200	3250	3550 (3318-3799)	3950	4000
Orotic acid	3000	3300	3990 (3500-4519)	4750	5000
Ascorbic acid	3500	3850	4100 (3832-4387)	4400	4500
Rutin	25,000	-	30,000	-	-
Galascorbine	3800	4050	4320 (4037-4622)	4650	4800

Shilov and T. I. Yakovlev (1964), in proposing a requirement for folic acid of 1-2 mg and for vitamin P of 50 mg, the therapeutic doses of these vitamins are, consequently, 10-40 mg and 500-1000 mg, respectively. Comparing the results obtained with those presented above, it must be noted that the therapeutic vitamin dosages are significantly less than their toxic doses. Thus, for vitamin B₁, the maximum therapeutic dose (1 mg/kg weight) is altogether 0.3% of the LD₅₀ obtained in the experiment, for vitamin B₂ 0.025%, nicotinic acid 0.65%, vitamin C 1.32%, vitamin B₆ 0.029%, folic acid 0.079%, galascorbine 0.45%, and rutin 0.07%. In calculating these figures we used the LD₅₀ of the vitamins for their intraperitoneal administration. Even if you take the maximum amount of the vitamins which shows a pharmacodynamic effect, for an average effective dose,

the therapeutic coefficient (ratio of the average lethal dose to the average effective dose) will in all cases exceed 100 (with the exception of ascorbic acid), which confirms the wide range of the therapeutic effect of vitamins. In addition, these data indicate the possibility of using vitamins for therapeutics in large doses.

In order to establish the optimal amount of vitamins for therapeutic application, the biological study of the effect of increased doses of these preparations is necessary above all. It is expedient to investigate the effect of significant doses of vitamins on the course of various pathological processes in the organism which are caused by extreme reactions. In this relation, the work of the Italian scientists is representative. In experiments on dogs, Barusco *et al.* (1968) formed conclusions about the usefulness for neuroleptanalgesia of thiamine and its monophosphoric ester, which have a narcotic effect in high dosages. Manami *et al.* (1968) administered thiamine internally in doses up to 250 mg/kg to patients at the time of narcosis and to healthy subjects - up to 350 mg/kg. Sauly *et al.* (1968) administered thiamine internally, on the average of 20 g to a man, in a 10% solution with the addition of 0.6 g of Pentothal. According to the data of Violani *et al.* (1968), 20 hours after administering 12-13 g of thiamine, it was not found in the blood.

According to our data, the doses of thiamine used by the Italian investigators are within the zone of toxic effect and could cause significant secondary effects.

The use, in anesthesiology, of thiamine, which has significant toxicity, indicates the possibility of increasing the therapeutic doses of other less toxic vitamin preparations within the zone of their nontoxic pharmacodynamic effect. However the use of increased doses of vitamin preparations must be substantiated on all sides.

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ИЗУЧЕНИЕ ТОКСИЧНОСТИ НЕКОТОРЫХ ВИТАМИННЫХ ПРЕПАРАТОВ В ЭКСПЕРИМЕНТЕ

Г. Н. Липкан, Киев

Витамины относятся к всесторонне изученным соединениям, однако в доступной нам литературе мы встретили лишь единичные работы, касающиеся изучения токсичности этих биологически активных веществ (А. М. Тимофеева, Г. А. Цофина, 1948; Я. Б. Максимович, 1963; Ю. М. Островский и соавт., 1963; Winter и соавт., 1967).

Нами изучена токсичность в острых опытах некоторых водорастворимых витаминов группы В, аскорбиновой кислоты, препарата витамина Р — рутина и галаскорбина, обладающего С- и Р-витаминной активностью. Опыты проведены на белых крысах, находившихся на обычном рационе питания. Токсичность определяли при помощи метода пребит-анализа, предложенного Литчфильдом и Вилкоксом (М. Л. Бельский, 1959). Во всех опытах определяли дозу витаминов, вызывающую гибель всех подопытных животных, и величину дозы, не вызывающей гибели. Для испытания каждой дозы брали 5 животных. Растворы препаратов готовили непосредственно перед введением на дистиллированной воде. Плохо растворимые вещества вводили в виде взвеси. Гибель животных учитывали в течение 2—3 дней. Полученные данные отражены в табл. 1 и 2.

Из всех изученных нами препаратов относительно высокой токсичностью обладает витамин В₁ и фолиевая кислота при внутривенном введении. Фосфорилированная форма тиамина — кокарбоксалаза в условиях опыта примерно в 5 раз менее токсична, чем сам витамин В₁.

В клинической практике все более широко используется фармакодинамическое действие витаминных препаратов. Однако повышенные дозы витаминов часто применяются без достаточных экспериментальных обоснований, исходя из эмпирических наблюдений и выводов, причем встречаются лишь единичные попытки каким-либо образом обосновать возможность применения повышенных доз витаминов с терапевтической целью. Э. Н. Малкин

Таблица 1. Острая токсичность витаминных препаратов при однократном внутривенном введении крысам (мг/кг)

Препарат	Максимально переносимая доза (ЛД ₅₀)	ЛД ₅₀	ЛД ₅₀ и ее доверительные пределы при р = 0,05	ЛД ₅₀	Абсолютно смертельная доза (ЛД ₁₀₀)
Витамин В ₁ (тиамин)	300	335	370 (312 ÷ 445)	475	500
Кокарбонизат	1600	1665	1935 (1745 ÷ 2150)	2500	2300
Витамин В ₂ (рибофлавин)	3000	—	> 4000	—	—
Пикотиновая кислота	700	820	1050 (900 ÷ 1255)	1700	1500
Витамин В ₆ (пиридоксин)	1500	1675	1925 (1750 ÷ 2118)	2105	2200
Фолиевая кислота	500	610	720 (600 ÷ 854)	820	600
Оротовая кислота	1500	1875	2200 (1880 ÷ 2574)	2675	2000
Аскорбиновая кислота	2100	2200	2459 (2131 ÷ 2774)	2810	2000
Рутин	15000	—	> 20000	—	—
Галаксорбин	2800	2850	31200 (2943 ÷ 3307)	3150	3500

(1952) считает, что для получения фармакодинамического эффекта витаминных препаратов необходимо применять дозы, в 10—20 раз большие физиологических, отражающих потребность человека в этих витаминах. Если исходить из физиологических норм суточной потребности человека в витаминах, утвержденных в 1960 г. Министерством здравоохранения СССР (Н. С. Ярусова, 1961), и вышеприведенного принципа расчета доз витаминных препаратов, обладающих фармакодинамическим эффектом, то для человека со средним весом 70 кг лечебные дозы находятся в пределах 20—60 мг для витамина В₁, 25—70 мг — для витамина В₂, 1000—2000 мг — для витамина С, 150—500 мг — для пикотиновой кислоты, 20—40 мг — для витамина В₆. По Н. И. Шилову и

Таблица 2. Острая токсичность витаминных препаратов при однократном подкожном введении крысам (мг/кг)

Препарат	Максимально переносимая доза (ЛД ₅₀)	ЛД ₅₀	ЛД ₅₀ и ее доверительные пределы при р = 0,05	ЛД ₅₀	Абсолютно смертельная доза (ЛД ₁₀₀)
Витамин В ₁ (тиамин)	700	760	840 (785 ÷ 899)	930	1000
Кокарбонизат	4000	4250	4650 (4116 ÷ 4976)	5170	5200
Витамин В ₂ (рибофлавин)	5000	—	8000	—	—
Пикотиновая кислота	2000	2350	2550 (2179 ÷ 2951)	3125	3200
Витамин В ₆ (пиридоксин)	2000	2750	3120 (2653 ÷ 3593)	3600	3500
Фолиевая кислота	3200	3370	3550 (3118 ÷ 3719)	3950	4000
Оротовая кислота	3000	3300	3500 (3040 ÷ 4739)	4750	5000
Аскорбиновая кислота	3500	3550	4100 (3852 ÷ 4357)	4350	4500
Рутин	25000	—	30000	—	—
Галаксорбин	3800	4050	4320 (4037 ÷ 4622)	4650	4800

Т. Н. Яковлеву (1964), предположительная потребность в фолиевой кислоте — 1—2 мг и в витамине Р — 50 мг, следовательно, терапевтические дозы этих витаминов составляют соответственно 10—40 и 500—1000 мг. Сравнивая полученные данные с приведенными выше, следует отметить, что терапевтические дозы витаминов значительно меньше их токсических доз. Так, для витамина В₁ максимальная терапевтическая доза (1 мг/кг веса) составляет всего около 0,3% от ЛД₅₀, полученной в эксперименте, для витамина В₂ — 0,025%, никотиновой кислоты — 0,65%, витамина С — 1,37%, витамина В₆ — 0,029%, фолиевой кислоты — 0,072%, аскорбиновой кислоты — 0,45%, рутина — 0,07%. При расчетах этих цифр мы пользовались ЛД₅₀ витаминов при их внутривенном введении. Если принять даже максимальные количества витаминов, оказывающие фармакодинамическое действие, за среднееэффективную дозу, терапевтический коэффициент (отношение среднесмертельной дозы к среднееффективной) будет во всех случаях превышать 100 (за исключением аскорбиновой кислоты), что свидетельствует о большой широте терапевтического действия витаминов. Кроме того, эти данные указывают на возможность применения витаминов с терапевтической целью в больших дозах.

Для того, чтобы установить оптимальные количества витаминов для применения их с терапевтической целью, прежде всего необходимо биологическое изучение действия повышенных доз этих препаратов. Целесообразно исследовать влияние значительных доз витаминов на течение различных патологических процессов в организме, вызываемых экстремальными воздействиями. В этом отношении показательны работы итальянских ученых. В опытах на собаках Вигиссо и соавт. (1968) сделали вывод о пригодности тиамин и его монофосфорного эфира, обладающих в высоких дозировках наркотическим действием, для нейролептанализа. Манчини и сопр. (1968) вводили тиамин внутривенно в дозе до 250 мг/кг больным во время наркоза внутривенно и здоровым испытуемым — до 350 мг/кг. Sauly и сопр. (1968) вводили внутривенно тиамин в среднем 20 г на человека в 10% растворе с добавлением 0,6 г тиопентала. По данным Violani и соавт. (1968), через 20 часов после введения 12—13 г тиамин последний в крови не обнаруживается.

По нашим данным, используемые итальянскими исследователями дозы тиамин находятся в зоне токсического действия и могут вызывать значительные побочные эффекты.

Применение в анестезиологии тиамин, обладающего значительной токсичностью, указывает на возможность потеснения терапевтических дозировок других, менее токсичных витаминных

препаратов в зоне их нетоксического фармакодинамического действия. Однако использование повышенных доз витаминных препаратов должно быть всемерно обосновано.

ON THE QUESTION OF THE COMPARATIVE TOXICITY OF THIAMINE AND CO-CARBOXYLASE

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Vitamin B₁ (thiamine) is one of the most widely studied vitamin preparations in experiments and clinical tests. The exchange processes which ensure the energy for the functional activity of the cardiovascular, nervous, and other systems of the organism are closely associated with thiamine metabolism. The preparation is appropriate for sedative action, for which it is widely used in neuritis not associated with a vitamin deficiency.

In connection with the wide use of thiamine, it is not possible to neglect the possibility of its metabolism in additional reactions associated with its preparation. In the medical literature, there have already been observed more than 200 cases of bad reactions to the administration of significant medicinal doses of vitamin B₁ to people (10). Dermatitis and allergenic reactions may occur after repeated administration of medicinal vitamin B₁ (4, 5, 9). In the literature (6), two cases are described of the occurrence of anaphylactic shock as a result of vitamin B₁ administration. In the world literature cases of sudden death after injection of vitamin B₁ (11, 12).


The toxicity of thiamine has been studied insufficiently in experiments, and in the literature studied, there are only isolated works which are devoted to this question. Winter and his co-workers (13) studied the toxicity of vitamin B₁ and two of its related substances. With internal administration to mice, a toxic dose which caused death in 50% of the animals (LD₅₀) was equal to 89 mg/kg and with oral administration, 8224 mg/kg.

It is evident that vitamin B₁ manifests its activity only in a phosphorylated form. Most often, especially in severe diseases of the heart and brain and hypoxia, the processes of phosphorylation of the vitamin are stimulated, which leads to a significant change in tissue metabolism even with sufficient introduction and absorption of the vitamin by the small intestine. In such cases, use of vitamin B₁ is recommended in its phosphorylated, cofermented form, co-carboxylase. Co-carboxylase is utilized in heart, neurological, metabolic, and other diseases.

Neuropathologically, co-carboxylase is utilized in treating scattered scleroses and polyneuritis; surgically it is used in cases of bad postoperative complications in acidosis. In gynecology, the preparation is used in uncontrollable vomiting of pregnancy, eclampsia, and other cases (7). A good medicinal effect is observed in the use of the preparation in intoxication of various origins and in comatose states. Co-carboxylase has naturally spasmolytic properties, and it improves blood supply to the myocardium (2). The preparation is effective in treating myocardial infarction, in a majority of cases of functional disruption causing fluttering of the heart muscles, during extrasystologia, tachycardia, and during fibrillation arrhythmia (1, 7). A series of uses of cardiac glycosides in women with cardiac decompensation suggests the use of co-carboxylase in order to normalize the metabolic processes in the myocardium. M. I. Egorov and his co-workers (7) observed that co-carboxylase did not cause similar phenomena and this is not considered a contraindication. Taking note of the fact that the toxicity

of vitamin B₁ preparations widely used in clinics has been insufficiently studied experimentally, we conducted comparative studies of the toxicity of thiamine and co-carboxylase, in acute investigations.

The toxicity studies of the preparations were conducted on half-grown animals of different sexes, which were kept under the same conditions on mixed food rations: mice (16-22 g in weight), rats (160-200 g), and rabbits (2000-2800 g). A pharmaceutical preparation of thiamine was used in the form of a powder, a co-carboxylase produced by the Moscow Experimental Vitamin Factory. The preparations were administered to the mice and rats interperitoneally, and to the rabbits internally. It is evident that with interperitoneal injection, the preparations are taken in rapidly, and their toxicity, which is indicated along the path of its introduction, approaches the toxicity value of that for internal administration. A solution was prepared in distilled water immediately before administration. Each dose of the preparation was used on not fewer than five animals. Indicators of toxicity were the general state of the animals under study, the time convulsions began, and the percentage of deceased. Observations of the animals were made for two days. Toxicity was studied by the probe analysis method proposed by Litchfield and Wilcoxon, using nomograms worked out by Z. Rot (3). The mean fatal dose and its probability were studied. The worth of the values of mean fatal dosage studied lies in the calculated probability, which reached 0.05. In studying the toxicity in all cases, the doses were found which caused death in all the animals studied, in absolute lethal dose (LD₁₀₀) and the size of the maximum bearable dose which caused no animal's death. The mean lethal dose (LD₅₀) was calculated on the basis of the criterion from evaluating thiamine and co-carboxylase toxicity.

With the simultaneous administration of thiamine interperitoneally, the LD₅₀ for mice was 495 (450-545) mg/kg. With a dose of 400 mg/kg, no animal died, but with administration of 600 mg/kg, all the animals died. The sensitivity of the rats to a toxic dose of thiamine did not differ much from the mouse sensitivity. LD₅₀ was 390 (342-445) mg/kg. In administration to rats interperitoneally of 300 mg/kg, no animal death was observed; with a 500 mg/kg dose all the animals died. Co-carboxylase appeared in our studies to be less toxic than thiamine. LD₅₀ with interperitoneal administration to mice was 1090 (973-1221) mg/kg. With a dose of 900 mg/kg, all the experimental animals remained intact; with 1300 mg/kg, the animals died. The rats were less sensitive to a toxic dose of co-carboxylase. LD₅₀ for interperitoneal administration was 1955 (1746-2190) mg/kg, the maximum bearable dose was 1600 mg/kg, and the absolutely fatal dose was 2300 mg/kg. The picture of the toxic dose of thiamine and co-carboxylase in the acute studies indicated injury to the central nervous system and respiration. The animals became depressed, adinamia developed, respiration was uneven, and *clonic*  -tonic spasms were often noted, especially with administration of fatal thiamine doses. The death of the animals, as a rule, took the form of respiration cessation; at the same time the heart still continued to beat for some time. In the animals which remained alive, paresis and paralysis of the extremities were often observed.

The picture of the toxic dose of the preparations was especially clearly observed in interperitoneal administration (in the area of the ear vein) in studies on rabbits. Even with doses which did not cause death in the animals, a significant acceleration of respiratory movement and spasms were observed. Very often, especially with the use of co-carboxylase, characteristic opisthotonos was observed suddenly after administration of the preparation, with the head thrown sharply back. This phenomenon called to mind in some measure the characteristic poses of doves with an expressed B₁ avitaminosis and polynauritis, which is described by the authors briefly and which indicate injury to the central nervous system. The average fatal dose for rabbits with interperitoneal administration of thiamine and co-carboxylase was 115 (74-178) and 510 (491-632) mg/kg, respectively. With a 40 mg/kg dose of thiamine, all the animals remained alive; with a 200 mg/kg dose, all the rabbits died from respiration cessation. With administration of 150 mg/kg, respiration curiously returned to some animals. With interperitoneal administration of 300 mg/kg of co-carboxylase, the animals remained uninjured. 700 mg/kg was an absolutely fatal dose.

Thus, in all the studies of acute toxicity, co-carboxylase was 2.2-5 times less toxic than thiamine. Symptoms of intoxication from co-carboxylase and thiamine in the acute studies were monotypic. It is possible that they are associated with the fact that with the administration of significant doses of co-carboxylase, metabolic dephosphorylation of the preparation is achieved.

The recommended daily dose of co-carboxylase with parenteral administration is 50 to 1000 mg (8). The thiamine dose with the same method of administration is 15 to 60 mg per day. Comparing these doses with the toxic doses of the preparations in the acute studies on animals, it was shown that the therapeutic doses in short periods of time are less toxic and the therapeutic coefficient (the ratio of average fatal dose to the curative dose) is equal, on the average, to 100-1250. But not considering that the doses of thiamine which are used in the clinic are 100 times less toxic, the toxic effects of thiamine in therapeutic administration occur all the more often in the extreme case. These are apparently associated with the fact that the nervous system in people is more sensitive to lower doses of thiamine than are animals. Comparisons of the acute toxicity of thiamine in experiments on animals and data from the literature on its toxic effects indicate the need for safe bounds on the maximum therapeutic doses of co-carboxylase in treating various diseases, derived from experimental studies of these doses. In our opinion, the maximum daily dose of co-carboxylase must be limited to 150-300 mg. Such a limitation is more in accordance with the data from a number of authors (7) on small doses, 50-100 mg per day, of co-carboxylase as the appropriate high dose in administration to people. The expressed effect of the curative dose of co-carboxylase in insignificant amounts is easily explained, since it is well-known that it is an integral part of the enzyme system, which explains the necessity for their dosages in trace amounts. Co-carboxylase belongs to the enzyme group.

Further toxicity studies on vitamin preparations corresponding to the requirements and methods of curative toxicology on different types of animals with different modes of administration and times

may yield additional practical medical information necessary for establishing the optimal curative doses of the preparations investigated.

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**ДО ПИТАННЯ ПРО ПОРІВНЯЛЬНУ ТОКСИЧНІСТЬ ТІАМІНУ
ТА КОХАРБОКСИЛАЗИ****Г. М. ЛІПКАН, М. П. ПАЩЕНКО***Київський інститут удосконалення лікарів*

Вітамін В₁ (тіамін) є одним з широко вивчених в експерименті і в клініці вітамінних препаратів. Обмінні процеси, які забезпечують енергією функціональну діяльність серцево-судинної, нервової та інших систем організму, тісно пов'язані з обміном тіаміну. Препарату властива седативна дія, і тому він широко застосовується при невритах, не зв'язаних з недостатком вітаміну.

У зв'язку з широким застосуванням тіаміну не можна забувати про можливість його передозування і побічних реакцій, зв'язаних з дією препарату. В медичній літературі вже відомо більш як 200 випадків захисних реакцій на введення звичайної лікувальної дози вітаміну В₁ людям (10). При лікуванні вітаміном В₁ після повторних введень можуть виникати дерматити та алергічні реакції (1, 5, 9). В літературі (6) описано два випадки виникнення анафілактичного шоку в результаті введення вітаміну В₁. У світській літературі відомі випадки з смертельним кінцем після ін'єкції вітаміну В₁ (11, 12).

Токсичність тіаміну в експерименті вивчена недостатньо, і в науковій

літературі є лише поодинокі роботи, присвячені цьому питанню. Вінтер і співробітники (13) зивчали токсичність вітаміну В₁ та двох його похідних. При внутрішньовенному введенні мишам токсична доза, яка викликала загибель 50% тварин (LD₅₀), дорівнювала 89 мг/кг, а при оральному введенні — 8224 мг/кг.

Відомо, що вітамін В₁ проявляє свою активність тільки в фосфорильованій формі. Дуже часто, особливо при тяжких захворюваннях серця, мозку, гіпоксії, процеси фосфорилування вітаміну порушуються, що приводить до значних змін тканинного обміну навіть при достатньому надходженні та всмоктуванні вітаміну кишечником. В таких випадках рекомендується застосування вітаміну В₁ у вигляді його фосфорильованої коферментної форми — кокарбоксилази. Кокарбоксилаза широко використовується при серцевих, неврологічних, обмінних та інших захворюваннях.

Невропатологи використовують кокарбоксилазу для лікування розсіяного склерозу, поліневритів, хірурги — у випадках важких післяопераційних ускладнень і шоків, в гінекології препарат застосовують при нестримному блюванні вагітних, еклампсії та в інших випадках (7). Добрий лікувальний ефект спостерігається від застосування препарату при інтоксикаціях різного походження та коматозних станах. Кокарбоксилазі притаманні спазмолітичні властивості, вона поліпшує кровопостачання міокарда (2). Препарат є ефективним засобом при лікуванні інфаркту міокарда, в більшості випадків функціональних розладів проведення збудження в серцевому м'язі, при екстрасистолії, тахікардії, при миготливій аритмії (1, 7). Поряд з використанням серцевих глікозидів у хворих з серцевою декомпенсацією доцільно застосування кокарбоксилази з метою нормалізації обмінних процесів у міокарді. М. І. Егоров з співавторами (7) вважають, що кокарбоксилаза не викликає побічних явищ і не має протипоказань до застосування. Беручи до уваги той факт, що токсичність препаратів вітаміну В₁, які широко застосовуються в клініці, експериментально досліджена недостатньо, нами було проведено порівняльне вивчення токсичності тіаміну і кокарбоксилази в гострих дослідках.

Вивчення токсичності препаратів проводилось на половозрілих тваринах різного полу, які утримувалися в однакових умовах на змішаному віварному раціоні на мишах (вага 16—22 г), щурах (вага 160—200 г), кролях (вага 2000—2800 г). Були використані античний препарат тіаміну у вигляді порошку та кокарбоксилаза виробництва Московського експериментального вітамінного заводу. Препарати вводили мишам і щурам внутрішньоочеревинно, кролям — внутрішньовенно. Відомо, що при внутрішньоочеревинних ін'єкціях препарати низько всмоктуються і їх токсичність, що визначається при цьому шляху введення, наближається до значень токсичності при внутрішньовенному введенні. Розчини готували на дистильованій воді безпосередньо перед введенням. Кожну дозу препарату досліджували не менш як на 5 тваринах. Показниками токсичності дії був загальний стан піддослідних тварин, їх поведінка, час початку корчів та процент загибелі. Спостереження за тваринами проводили протягом двох днів. Токсичність визначали за допомогою методу пробіг-аналізу, запропонованого Літчфілдом і Уїлкоксом по номограмах, розроблених З. Ротом (3). Визначали середню смертельну дозу та її довірчі межі. Вірогідність того, що справжнє значення величини середньосмертельної дози, що визначається, знаходиться поза врахуванням довірчих меж, дорівнювала 0,05. При визначенні токсичності в усіх випадках знаходили дози, що викликали загибель всіх піддослідних тварин — абсолютно смертельну дозу (LD₁₀₀) і величину максимально переносної дози, яка не викликала загибелі тварин. Основним критерієм для оцінки токсичності тіаміну та кокарбоксилази була середня смертельна доза (LD₅₀).

При одноразовому введенні тіаміну LD_{50} для мишей при внутрішньоочередовому введенні дорівнювала 495 (450 + 545) мг/кг. При дозі 400 мг/кг жодна тварина не загинула, а при введенні 600 мг/кг загинули всі тварини. Чутливість пацюків до токсичної дії тіаміну не набагато відрізнялася від чутливості мишей. LD_{50} дорівнювала 390 (342 + 445) мг/кг. При введенні пацюкам внутрішньоочередово 300 мг/кг ми не спостерігали загибелі тварин, при дозі 500 мг/кг вagi всі тварини гинули. Кокарбоксілаза в наших дослідках виявилася менш токсичною, ніж тіамін. LD_{50} при внутрішньоочередовому введенні мишам дорівнювала 1090 (973 + 1221) мг/кг. При дозі 900 мг/кг всі піддослідні тварини залишилися непошкодженими, при 1300 мг/кг — тварини загинули. Пацюки були менш чутливими до токсичної дії кокарбоксілази. LD_{50} при внутрішньоочередовому введенні — 1935 (1735 + 2150) мг/кг, максимально перенесена доза — 1600 мг/кг, абсолютно смертельна — 2300 мг/кг. Картина токсичної дії тіаміну та кокарбоксілази в гострих дослідках вказувала на пошкодження центральної нервової системи та дихання. Тварини ставали припиненими, у них розвивалася адинамія, розлад дихання, часто спостерігалися клякко-тонічні корчі, особливо при введенні смертельних доз тіаміну. Смерть тварин, як правило, наставала під припинення дихання, у той час як серце ще на протязі деякого часу продовжувало скорочуватись. У тварин, що залишилися живими, часто спостерігалися парези та параліти кінцівок.

Особливо виразно можна було спостерігати картину токсичної дії препаратів при їх внутрішньовенному введенні (в краєву вену уха) і дослідках на кролях. Навіть при дозах препаратів, які не викликали загибелі тварин, спостерігалося значне прискорення дихальних рухів та судороги. Дуже часто, особливо при експерименті кокарбоксілази, спостерігався характерний опістотонус відразу ж після введення препарату, з різким відкиданням голови назад. Це явище в деякій мірі нагадувало характерні позн голубів при вираженому В₁ авітамінізмі та поліневриті, які описані багатьма авторами і вказують на пошкодження центральної нервової системи. Середня смертельна доза для кролів при внутрішньовенному введенні тіаміну та кокарбоксілази дорівнювала відповідно 115 (74 + 178) мг/кг та 510 (491 + 652) мг/кг. При дозі тіаміну 40 мг/кг усі тварини залишилися живими, при дозі 200 мг/кг всі кролі гинули від припинення дихання. При введенні 150 мг/кг штучне дихання повертало деяких тварин до життя. При внутрішньовенному введенні 300 мг/кг кокарбоксілази тварини залишалися непошкодженими. 700 мг/кг є абсолютно смертельною дозою.

Таким чином, в усіх дослідках при вивченні гострої токсичності кокарбоксілази була в 2,2-5 разів менш токсичною, ніж тіамін. Симптоми інтоксикації кокарбоксілазою та тіаміном в гострих дослідках були однотипними. Можливо, це зв'язане з тим, що при введенні значних доз кокарбоксілази відбувається метаболічне дефосфорилювання препарату.

Рекомендованою добовою дозою кокарбоксілази при парентеральному введенні є доза від 50 до 1000 мг (3). Доза тіаміну при такому ж методі введення коливається від 15 до 60 мг на людину. Порівняння цих доз з токсичними дозами препаратів в гострих дослідках на тваринах показало, що терапевтичні дози в багато разів менш токсичні і терапевтичний коефіцієнт (відношення середньосмертельної дози до лікувальної) дорівнює, в середньому, 100-1200. Але, незважаючи на те, що дози тіаміну, які застосовуються в клініці, в сотні разів менші токсичних, токсичні ефекти тіаміну при введенні терапевтичних доз останнього транзитують все частіше. Це, очевидно, зв'язано з тим, що нервова система людини чутливіша до дії тіаміну, ніж у тварин. Порівняння гострої токсичності тіаміну з експерименту на тваринах і даних літератури про його токсичні ефекти вказує на необхідність пев-

ного обмеження максимальної терапевтичної дози кокарбоксилази при лікуванні різних захворювань, виходячи з визначення її дії, в експерименті. На нашу думку, максимальну добову дозу кокарбоксилази необхідно обмежити 150—200 мг. Таке обмеження тим більш доцільне, що за даними ряду авторів (7) вже в невеликих дозах — 50—100 мг на добу — кокарбоксилазі властиві високі терапевтичні якості при одноразовому введенні людині. Виражений ефект лікувальної дії кокарбоксилази в незначних кількостях легко пояснюється, так як добре відомо, що коензими, які беруть безпосередню участь в різних видах обміну, будучи складовою частиною ферментних систем, необхідні для виявлення їх дії в слідових кількостях. І кокарбоксилаза належить до коферментів.

Дальше вивчення токсичності вітамінних препаратів відповідно вимогам і методам лікарської токсикології на різних видах тварин при різних шляхах введення і строках може дати практичній медицині додаткові відомості, необхідні для встановлення оптимальних лікувальних доз досліджуваних препаратів.

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ON THE COMPARATIVE TOXICITY OF THIAMINE AND COCARBOXYLASE

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SUMMARY

A study is presented of the acute toxicity of thiamine and its phosphorylated form (cocarboxylase) in acute experiments on different kinds of animals (mice, rats, rabbits). It was found that cocarboxylase proved 2—5 times less toxic than thiamine.

The authors recommend to restrict the maximum daily dose of cocarboxylase to 150—200 mg and suggest the necessity of further investigations of the toxic effects of vitamin preparations with the purpose of establishing optimal therapeutic doses.

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Thiamine Influence Upon Laxative Action*

By

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NEW YORK, NEW YORK

THE animal experiments (1, 2), by showing that the beneficial action of thiamine upon the intestine appears to be limited to hypothiaminotic atony, justifies the use of thiamine in conditions having this etiology. There is, however, a tendency to use thiamine also in combination with cathartics. The question whether there is a synergism between thiamine and laxatives, may be answered by experiments in the rhesus monkey, the only species suitable for measuring the effectiveness of cathartic drugs (3, 4).

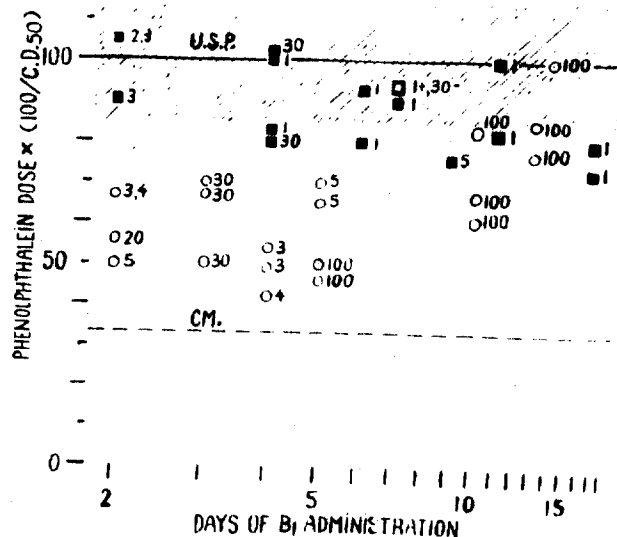
This report is based upon 443 experiments in rhesus monkeys.† Phenolphthalein was chosen as a test substance because the response of the monkey to this drug is well defined and an assay method is available (4, 5, 6). Eleven adult monkeys of either sex were calibrated with a standard U.S.P. phenolphthalein in 14 to 65 (average 38, total 413) experiments to determine the individual oral threshold cathartic dose (C.D.₅₀). In 25 experiments the same monkeys were fed varying fractions of the individual C.D.₅₀, and at the same time daily doses of one to 100 mg./kg. of thiamine hydrochloride were administered in two equal doses in 8 hours. The period of thiamine treatment extended over the day the phenolphthalein was administered, the subsequent day, and a varying number of preceding days. The care of the monkeys, diet, administration of the drugs, recording and evaluation of the laxative effect were described elsewhere (4, 5, 6, 7).

A survey of the experiments is given in graph 1, representing the distribution of positive and negative results in relation to the phenolphthalein dose and to the period and dosage of the thiamine treatment. When the individual sensitivity to the laxative changed in the course of the long calibration period, the test dose given in the thiamine period was referred to the C.D.₅₀ of the pro- and that of the recalibration period.

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†Sponsored by Elix, Inc., Brooklyn, N. Y.

Hence, the number of entries in the graph is higher than the number of experiments.

In the evaluation proper, these variations in sensitivity did not interfere with the result since it is the express purpose of the "procedure of approximation" (5, 6, 7) to take them into consideration. By approxi-



■ experiments with, ○ experiments without laxative effect. — Figure at individual experiment — thiamine doses in mg. kg. per day.

mation from a total of 40 values of maximum and minimum potency obtained in the 25 synergism experiments the potency of phenolphthalein was found to be 1.175 ± 0.20 as referred to the potency value 1.0 of phenolphthalein in the calibration periods. In other words, the synergistic increase in potency of phenolphthalein effected by all kinds of thiamine treatment

amounted to about 18 per cent, with a coefficient of variation of \pm of 17%.

In the routine bioassay of cathartics in rhesus monkeys, the range of variation is about \pm 10 per cent. It may be assumed to be greater in experiments of the type presented here because both pre- and re-calibration experiments are separated from the test experiment by greater intervals of time, the former by the thiamine pre-treatment period, the latter by a period of safety required to avoid possible persistence of thiamine effect. An even wider range than \pm 17 per cent, but with a mean value closer to 1.0, might have been obtained by conducting additional experiments with phenolphthalein doses higher than the C.D....

1.175 is the mean value of potency from experiments with widely varying thiamine treatment. As can be seen from the graph, there is, however, no convincing indication that the effectiveness of phenolphthalein was significantly increased by prolonged thiamine treatment or by higher thiamine dosage. To the contrary, if there was any synergistic influence in relation to the thiamine dosage, it was rather in the range of the smaller doses. 30 to 100 mg. kg. daily are considerably greater than a dosage of maintenance or of complete substitution. That these large doses failed to increase the effectiveness of the laxative, indicates in itself that diet, food intake and state of health of the animals were of no great importance. Actually, no more convincing synergistic effect of thiamine than in normal animals was seen in monkeys with a fatal disease (usually tuberculosis), with lower sensitivity to the laxative, or with decreasing body weight, three possible signs of hypothiaminosis. For

practical purposes, the insignificance of the synergism attained in these thiamine experiments can best be emphasized by contrasting it with the incomparably greater increase in effectiveness, namely by 200 per cent, which is readily obtained by replacing U.S.P. phenolphthalein by commercial—so-called “yellow”—phenolphthalein.

Since in some of these experiments any indication of enhanced intestinal activity was absent even after thiamine doses several hundred times higher than those in therapeutic use, their outcome is direct evidence that thiamine is useless as a laxative and has no place in the therapy of constipation “unless it is the direct result of a deficiency” (8).

SUMMARY

Experiments in the rhesus monkey give no support to the assumption of a synergistic influence of Vitamin B₁ upon laxative action. Daily doses from 1.0 to 100 mg. kg. thiamine hydrochloride, orally administered over periods of 2 to 17 days, did not significantly increase the effectiveness of a laxative (phenolphthalein). The average increase in effectiveness in 25 experiments was 18 per cent with a variation of \pm 17 per cent.

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Question on the toxicity of Diphosphothiamine.

A. Malachovskis.

Vitamin B₁ or thiamine has found wide recognition in medical and veterinarian therapy for a variety of ailments or to induce a body to grow. Sound toxicological research has shown that large thiamine doses are active similar to tubocurarine and cause bulbar breathing centrum paralysis. It has been shown that death causing doses of thiamine for mice are 125 mg/kg; rat, 250 mg/kg; and rabbits, 300 mg/kg body weight (1). Other authors' research show that intravenously injected thiamine induces death (lethal dose is 125-350 mg/kg body weight), injected subcutaneously the lethal dose is six times larger, but injected per os, it is increased a hundred times(2). In scientific references we find explanations that use of large doses of thiamine for human therapy sometimes causes intoxication symptoms: changes in breathing, mild irritability, vomiting, tachycardia, headaches; and sometimes show allergic symptoms - swelling, rash, eosinophilia. A few lethal cases when using thiamine for treatment of illness are described(1,3).

Since the years 1935-1938, it has been known that in human or animal body, thiamine changes to ester-thiamine pyrophosphate, or diphosphothiamine, and then in this

form participates in numerous biochemical reactions. In the present time diphosphothiamine is clinically adopted in foreign countries and the Soviet Union, but until now our search of the scientific literature has found little information on the toxicity of this preparation.

In the year 1954 of our dissertation work (4), it was shown that small doses of diphosphothiamine do not cause any toxic disorder when the preparation is injected subcutaneously or intravenously to humans, rats, white mice, and rabbits. At that time diphosphothiamine doses of 0.15-5.0 mg/kg body weight were used, however we did not get a wider understanding of this preparation precise toxicity.

In 1954 diphosphothiamine was tried in complementary therapy of diabetes, the dose was 2-6 mg intramuscular, and no negative reactions were observed(5). Later in the treatment of people afflicted with disseminate sclerosis, the diphosphothiamine dose was increased to 50 mg, and even when injected intravenously , it surprisingly did not cause any stress or symptoms of toxicity(6). Lately, V. Kutorgos published very precise research work (7). The author underlines that diphosphothiamine has less toxicity than thiamine. Its maximal tolerant doses for mice are 350 mg/kg, but absolutely lethal dose is more likely to

be 450 mg/kg body weight when the preparation is injected subcutaneously or intraperitoneally. The author did not verify the toxicity of diphosphothiamine when injected intravenously. For this reason we desire to supplement this lack of information with our research work, and would like to make known the results of our specific research.

Research was done in two series: February 27 - March 25, 1951, and April 28 - June 20, 1952. For research 330 white mice were used (165 males and 165 females) with a body weight approximately 16-18 g. The mice were raised by the author himself and all the time lived in normal vivarium conditions. Research was performed in groups of 10 each (5 males and 5 females). Laboratory animals were injected intravenously with sterile solutions of thiamine, diphosphothiamine, and a mixture of equal parts. The concentration of the solutions was 0.2%; thiamine solution was sterilized in the autoclave at 1.5 atmosphere for twenty minutes, and the diphosphothiamine solution was sterilized by filter sterilization using a Zeico filter. In the experimental investigations, 30, 60, 90, 120, 150, 200, and 250 mg/kg doses were used. We have to explain that in the experiment using the 250 mg/kg dose, we had to inject approximately a two milliliter mixture; which we must stress, this dosage caused additional trauma for the experimental laboratory animals. Toxicological research results were evaluated

with Fisher's formula (8). Animals injected with a dose of 30 mg/kg of thiamine, diphosphothiamine, or their mixture did not show any noticeable toxic symptoms. Mice injected with a dose of 60 mg/kg thiamine were disturbed by small convulsions and immediately returned to normal; diphosphothiamine and their mixture of both did not cause any stress. The same was repeated with a dose of 90 mg/kg body weight; the thiamine group has shown much deeper convulsions, and one mouse was very irritated until after two hours when her condition cleared. Further observation of the animals for five to seven days after the experiment did not show any dead and their behaviour was normal.

The first genuine toxic symptoms and death accidents were observed after injecting 120 mg/kg thiamine. Immediately after injection of the preparation, two animals died of convulsions and disturbed breathing. The others reacted with convulsions and laying on their side; however, after one hour and fifteen minutes the animals improved and after five days their discomfort disappeared. After injecting this same dose of diphosphothiamine and a mixture of the two, small convulsions were observed between thirty to thirty-five minutes which later disappeared and the animals did not appear to differ from the other controls. Therefore, the maximal tolerant dose of thiamine injected intravenously

could be 100 mg/kg body weight.

In the first research series the mice were injected intravenously with thiamine, 150 mg/kg, which caused all the mice to die. In the second research series, six mice died immediately with paralysis, convulsions and disturbed breathing symptoms. After thirty to thirty-five minutes the next two mice died in grave discomfort, and after two and one-half hours two mice began to recover and after a period of five days returned to normal. Both preparations mixed and injected in this dosage caused immediate death of two mice, and after thirty minutes again one mouse died. Other animals reacted with the described symptoms and after two hours, their condition returned to normal.

A thiamine dose of 200 mg/kg was absolutely lethal, and in minutes after injection, all animals died with the same symptoms. After injection with diphosphothiamine all mice reacted violently, with convulsions, laying on the side, and paralysis. In the first research series, four died and in the second, three animals. After three hours the others began to feel better and after a period of five days their condition became normal. After an injection of the mixture of both, all animals reacted very violently and six mice died with described symptoms. The others conditions improved after six hours and they slowly returned to their normal life.

However an injection of 250 mg/kg thiamine caused death of all animals with the usual symptoms. This same dose of diphosphothiamine led to the death of eight animals during a few minutes, and again the death of five after a period of three hours. After four to six hours the other mice began to recover very slowly and definitely reached a normal condition after two days. During the remaining observation time, they lived normally. When a mixture of both preparations was injected, half of the animals died within fifteen minutes, the remaining five reacted very seriously: four died in a period of two and one-half hours, and the last mouse died after twelve hours.

Final toxicological research results are described in chart number one. The results of our work indicates that diphosphothiamine is less toxic than thiamine. The toxicity of the diphosphothiamine and thiamine mixture places it between the two preparations when used separately.

Thiamine, diphosphothiamine and their mixture influence on mouse mortality.

Preparations	Number of animals	30 mg/kg		60 mg/kg		90 mg/kg		120 mg/kg		150 mg/kg		200 mg/kg		250 mg/kg	
		died	left	died	left	died	left	died	left	died	left	died	left	died	left
Thiamine (T)	130	0	10	0	20	0	20	4	16	18	2	20	0	20	0
Diphosphothiamine (DFT)	130	0	10	0	20	0	20	0	20	3	17	7	13	13	7
T & DFT	70	0	10	0	10	0	10	0	10	3	7	6	4	10	0

Analysis of our research shows that the maximal tolerant thiamine dose injected intravenously is equal to about 100 mg/kg, Diphosphothiamine and a mixture of both is closer to 140 mg/kg body weight. The latest research shows that the absolute lethal dose of thiamine for mice is equal to about 200 mg/kg, diphosphothiamine more than 250 mg/kg, and a mixture of both, 250 mg/kg. In our opinion this lesser toxicity of diphosphothiamine can be explained by the fact that diphosphothiamine is the psychological form of thiamine in the animal organism, and we do not need mobilization of a special mechanism for thiamine activation. Some authors (1,2,3) point out the possibility that diphosphothiamine passes through membranes slower than thiamine. Our many years of research shows the very important influence of diphosphothiamine on carbohydrates and it causes some

other biochemical changes visible after five minutes after the preparation injection intravenously or subcutaneously. For this reason in our opinion, the lesser toxicity of diphosphothiamine can not be explained by its lesser reabsorption than thiamine.

Statistically evaluated results of interval dose of 150-200 mg/kg body weight of diphosphothiamine and its mixture with thiamine indicates that diphosphothiamine is less toxic than thiamine (x^2 between 5.0 to 10.8). The conclusion from our basic work is very timely because it proves that diphosphothiamine not only has excellent activity, and less toxicity than thiamine, but also has a future in the clinical usage of this preparation for internal diseases therapy.

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BIOLOGIJA, V. 1965

DIFOSFOTIAMINO TOKSIŠKUMO KLAUSIMU

A. MALACHOVSKIS

Vitaminas B₁, arba tiaminas, rado platų pritaikymą medicinoje ir veterinarijoje įvairiems susirgimams gydyti bei augimui skatinti. Gilūs toksikologiniai tyrimai parodė, kad didelės tiamino dozės veikia analogiškai tubokurarinui, sukeldamos bulbarinio kvėpavimo centro paralyžių. Nurodoma, kad mirtinos tiamino dozės pelėms yra 125 mg/kg, žiurkei – 250 mg/kg, triušiiui – 300 mg/kg kūno svorio [1]. Kiti autoriai nurodo, kad į veną įšvirkštas tiaminas sukelia mirtį (mirtina dozė 125–350 mg/kg kūno svorio), įšvirkščiant po oda, mirtina dozė yra 6 kartus didesnė, o įšvirkščiant per os, ji padidėja 100 kartų [2]. Literaturoje randame nurodymų, kad, naudojant žmonėms gydyti dideles tiamino dozes, kartais esti intoksikacijos simptomų: pakinta kvėpavimas, atsiranda pykinimas, vėmimas, tachikardija, galvos svaigimas, kartais būna alerginiai reiškiniai – patinimas, išbėrimas, eozinofilija. Aprašyti taip pat keli žmonių mirties atvejai, panaudojus tiaminą ligonių gydymui [1, 3].

Tačiau nuo 1935–1938 m. žinoma, kad žmogaus bei gyvulių organizme tiaminas virsta esteru – tiamino pirofosfatu, arba difosfotiaminu, ir tokioje formoje dalyvauja įvairiose biocheminėse reakcijose. Dabartiniu metu difosfotiaminas rado pritaikymą žmonių klinikoje užsienyje ir Tarybų Sąjungoje, tačiau mums prieinamoje literaturoje radome mažai duomenų apie šio preparato toksiškumą. Dar 1951 m. mūsų disertaciniame darbe [4] buvo nurodyta, kad mažos difosfotiamino dozės nesukelia jokių toksinių reiškinių, įšvirkštus preparatą po oda arba į veną žmonėms, žiurkėms, baltosioms pelėms ir triušiams. Tačiau mūsų tada buvo naudojamos 0,15–5,0 mg/kg kūno svorio difosfotiamino dozės ir jos negalėjo giliau apibūdinti šio preparato toksiškumo.

1954 m., taikant difosfotiaminą kompleksiniam diabetikų gydymui, buvo panaudotos 2–6 mg preparato dozės į raumenis, ir jokių neigiamų reiškinių nebuvo pastebėta [5]. Vėliau, gydant difosfotiaminu sergančius diseminuota skleroze, preparato dozės buvo padidintos iki 50 mg ir, nežiurint jo įšvirkštimo į veną, taip pat nebuvo pažymėta toksinių reiškinių [6]. Pastaruoju metu buvo paskelbti nuodugnūs V. Kutorgos [7] tyrimai. Autorius pažymi, kad difosfotiaminas yra mažiau toksiškas, negu tiaminas. Jo maksimali tolerantiinė dozė pelėms yra 350 mg/kg, o absoliuti mirtina dozė – daugiau kaip 450 mg/kg kūno svorio, įšvirkščiant preparatą po oda arba į pilvo ertmę. Tačiau autorius nepatikrinio sulėisto į veną difosfotiamino toksiškumo ir dėl to, norėdami užpildyti šią spragą, šiame darbe pateikiame atitinkamų tyrimų rezultatus.

Bandymai buvo daryti dviem serijomis. 1951 m. vasario 27–kovo 25 dienomis ir 1952 m. balandžio 28–birželio 20 dienomis. Bandymams panaudota 330 baltųjų pelių (165 patinų ir 165 patelių), kurių kūno svoris svyravo nuo 16 iki 18 g. Pelės buvo paties autoriaus išaugintos ir visą

aišką gyvenimą normaliose vivariumo sąlygose. Bandymai buvo atliekami grupėmis – po 10 gyvuliukų grupėje (5 patinai, 5 patelės). Gyvuliukams į veną buvo švirkščiami tiamino, difosfotiamino bei jų mišinio lygiomis dalimis sterilūs tirpalai. Tirpalų koncentracija – 0,2%; tiamino skiedinys buvo sterilinamas autoklave 1,5 atm. 20 min., difosfotiamino tirpalas sterilinamas, filtruojant per Zeico filtrą. Bandymuose buvo išširtos 30, 60, 90, 120, 150, 200, 250 mg/kg kūno svorio preparato dozės. Tenka pabrėžti, kad, naudojant bandymuose 250 mg/kg dozę, teko įšvirkšti apie 2 ml skiedinio, kas, žinoma, sudarė tam tikrą papildomą traumą eksperimentiniams gyvuliukams. Toksikologinių tyrimų rezultatai buvo apdoroti statistiškai pagal Fišerio formulę [8]. Įšvirkštus gyvuliukams tiaminą, difosfotiaminą ar jų mišinį dozėje 30 mg/kg kūno svorio, jokių intoksikacijos reiškinių nepastebėta. 60 mg/kg tiamino dozė kai kurioms pelėms sukėlė nežymius ir greit pranykstančius traukulius, difosfotiaminas bei preparatų mišinys jokių reiškinių nepadarė. Tas pats pasikartėjo, esant dozei 90 mg/kg kūno svorio, tačiau tiamino grupėje traukuliai buvo daug sunkesni, o viena pelytė buvo sunkioje būklėje ir pasitaisė tik po 2 val. Toliau stebint gyvuliukus artimiausių 5–7 dienų bėgyje po bandymo, mirties atvejų nebuvo, jų laikysena buvo normali.

Pirmieji ryškūs toksiniai reiškiniai ir mirties atvejai buvo pastebėti, įšvirkštus 120 mg/kg tiamino. Tuojau po preparato suleidimo žuvo 2 gyvuliukai, esant kloniniams traukuliams ir sutrikus kvėpavimui. Kiti reagavo traukuliais, šonine padėtimi, tačiau po 1 val. 15 min. gyvuliukai pasitaisė ir artimiausių 5 dienų bėgyje jų laikysena buvo normali. Suleidus jiems tokią pat dozę difosfotiamino arba abiejų preparatų mišinio 0–35 min. laikotarpyje, buvo pažymėti nežymūs traukuliai, kurie vėliau praėjo, ir gyvuliukai nesiskyrė nuo kontrolinių. Tokiu būdu maksimali tolerantiinė tiamino dozė po preparato įšvirkštimo į veną gali būti prilyginta apytikriai 100 mg/kg kūno svorio.

Suleidus pelėms į veną 150 mg/kg tiamino, pirmoje bandymų serijoje visos jos žuvo. Kitoje bandymų serijoje žuvo tuojau po suleidimo 6 pelės, esant galūnių paralyžiaus, traukulių bei kvėpavimo sutrikimo reiškiniams. Po 30–35 min. žuvo dar 2 pelės, iki tol buvusios labai sunkioje būklėje. 2 pelytės po 2,5 val. pradėjo taisyti ir vėliau 5 dienų bėgyje jų laikysena buvo normali. Suleidus gyvuliukams tokią pat dozę difosfotiamino, pirmoje serijoje žuvo 2, o antroje – 1 pelytė. Kiti gyvuliukai reagavo traukuliais, šonine padėtimi, užpakalinių galūnių parezais, tačiau vėliau pasitaisė ir gyveno normaliai. Suleidus gyvuliukams 150 mg/kg abiejų preparatų mišinio, tuojau po injekcijos žuvo dvi pelytės, po 30 min. dar viena. Likusios reagavo jau aprašytais simptomais, bet po 2 val. pasitaisė ir vėliau jų laikysena buvo normali.

Tiamino dozė 200 mg/kg kūno svorio buvo absoliučiai mirtina ir artimiausiomis minutėmis po injekcijos visi gyvuliukai žuvo, esant jau aprašytiems simptomams. Po difosfotiamino suleidimo visos pelytės reagavo traukuliais, šonine padėtimi, parezais. Pirmoje bandymų serijoje žuvo 4, kitoje – 3 gyvuliukai. Likusieji po 3 val. pradėjo taisyti ir paskui 5 dienų bėgyje jų laikysena buvo normali. Po preparatų mišinio suleidimo visi gyvuliukai sunkiai reagavo, esant aprašytiems reiškiniams, ir galandose bėgyje žuvo 6 pelytės. Likusios po 6 val. pasitaisė ir vėliau gyveno normaliai.

Pagaliau, suleidus pelėms į veną 250 mg/kg tiamino, visos žuvo tuojau po injekcijos, esant įprastiesiems reiškiniams. Tokia pat dozė difosfotiamino sukėlė artimiausių kelių minučių bėgyje 8 gyvulių mirtį, dar 5 žuvo artimiausių 3 val. laikotarpyje. Likusios pelės 4–6 val. pradėjo taisyti, tačiau dar sekančią parą jos buvo mažiau judrios ir galūnais pasitaisė

tik po 2 parų. Likusį stebėjimo laiką jos gyveno normaliai. Suleidus pelėms preparatų mišinio, artimiausių 15 min. bėgyje pusė jų žuvo, likusios 5 reagavo labai sunkiai ir 4 krito 2,5 val. bėgyje, o paskutinė – po 12 val.

Galutinai toksikologinių tyrimų rezultatai yra apibendrinti 1 lentelėje. Iš lentelės duomenų aiškiai matyti, kad difosfotiaminas pasižymi žymiai mažesniu toksiškumu, negu tiaminas. Timino ir difosfotiamino mišinys užima toksiškumo atžvilgiu tarpinę padėtį tarp abiejų preparatų, paimtų atskirai.

1 lentelė

Timino, difosfotiamino ir jų mišinio įtaka pelių mirtingumui

Preparatas	Gyvuliukų skaičius	30 mg/kg		60 mg/kg		90 mg/kg		120 mg/kg		150 mg/kg		200 mg/kg		250 mg/kg	
		krito	liko	krito	liko	krito	liko	krito	liko	krito	liko	krito	liko	krito	liko
Tiaminas (T)	130	0	10	0	20	0	20	4	16	18	2	20	0	20	0
Difosfotiaminas (DFT)	130	0	10	0	20	0	20	0	20	3	17	7	13	13	7
T+DFT	70	0	10	0	10	0	10	0	10	3	7	6	4	10	0

Mūsų tyrimai rodo, kad maksimali tolerantiinė timino dozė, švirkšdiant jį į veną, yra lygi apie 100 mg/kg svorio, difosfotiamino ir abiejų preparatų mišinio ji artima 140 mg/kg kuno svorio. Tolesni tyrimai parodė, kad absoliuti letaliinė timino dozė pelėms yra lygi apie 200 mg/kg, difosfotiamino – daugiau kaip 250 mg/kg, o abiejų preparatų mišinio – 250 mg/kg kuno svorio. Toks mažesnis difosfotiamino toksiškumas, mūsų nuomone, gali būti paaiškintas tuo, kad jis sudaro fiziologiškai aktyvią timino formą ir gyvuliuko organizmui nereikia mobilizuoti atitinkamų mechanizmų timino suaktyvinimui. Kai kurie autoriai [1, 2, 3] nurodo, kad, matomai, difosfotiaminas per membranas praeina sunkiau, negu tiaminas. Mūsų ilgamečiai tyrimai rodo, kad labai žymus difosfotiamino poveikis karboanhidrazės ir kai kurių kitų biocheminių rodiklių pakitimui konstatuojamas jau po 5 min., suleidus preparatą į veną arba po oda. Dėl to, mūsų nuomone, mažesnis difosfotiamino toksiškumas negali būti paaiškintas jo mažesne rezorbcija, palyginus su tiaminu.

Statistinis gautų rezultatų apdorojimas parodė, kad dozių intervale 150–200 mg/kg kuno svorio difosfotiaminas ir jo mišinys su tiaminu pasižymi mažesniu toksiškumu, negu tiaminas (χ^2 svyruoja nuo 5,0 iki 10,8). Ši pagrindinė mūsų darbo išvada yra labai aktuali, nes tuo įrodoma, kad difosfotiaminas turi pranašumą prieš tiaminą ne vien tik savo veiklumu, bet ir toksiškumu, ir sudaro perspektyvų preparatą vidaus ligų klinikoje.

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К ВОПРОСУ О ТОКСИЧНОСТИ ДИФОСФОТИАМИНА

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Резюме

Как известно, тиамин в организме человека и животных подвергается фосфорилированию и в форме дифосфотиамина принимает участие в обменных процессах. В настоящее время дифосфотиамин (кокарбоксилаза) нашел широкое применение в клинике, однако в литературе вопрос о токсичности этого препарата освещен недостаточно. В настоящей работе отражены результаты наших исследований, проведенных в 1951—1952 гг., в которых сравнивалось токсическое действие тиамина, дифосфотиамина и их смеси в равных количествах на организм белых мышей. Препараты применялись однократно в виде внутривенных вливаний, в дозе 30—250 мг/кг веса тела. В результате проведенных опытов выявлено, что максимальная толерантная доза тиамина составляет около 100 мг/кг, для дифосфотиамина и смеси препаратов она составляет около 140 мг/кг. Абсолютная смертельная доза для тиамина — около 200 мг/кг, для дифосфотиамина — выше 250 мг/кг, для смеси препаратов — 250 мг/кг. Таким образом, можно заключить, что дифосфотиамин обладает меньшей выраженной токсичностью, чем тиамин, а смесь обоих препаратов занимает промежуточное положение.

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Allergic diseases are an expression of a reaction occurring between an antigen and an antibody, a reaction which arises because of the action of the antigen. The kind and degree of intensity of this reaction depends on the reactivity of the organism, which may vary. If it is greater than average, it may produce allergic symptoms. It is significant that with high reactivity of the allergic organism, nonspecific stimuli may also evoke this same allergic reaction. As a result of this, the picture of allergic diseases is varied.

Allergic diseases of the nervous system are characteristic in having different features for the polymorphic forms and various courses of the allergy. The allergic reaction may affect the tissues of the central nervous system, the peripheral nerves, and the medulla meninx (2,3).

The case presented by us seems interesting from the point of view of etiology, especially the severe course of the disease, and skin changes which call to mind a drug rash.

DESCRIPTION OF THE CASE

S. J., 25 years old (illness history #789.69), a director in a factory, was directed to the clinic on August 6, 1969 from the county hospital with suspected encephalitis of the brain meninx in the course of measles.

Ill for a month, he suffered a head injury from loss of consciousness in a highway accident. As a result of a continuous head pain, he was treated for seven days in the hospital and then in the Provincial Neurological Clinic. On July 26, 1969 he felt ill, pain occurred in the pit of his stomach, and a bright red rash appeared on the skin of the stomach and rib cage. After two days, the patient was running a fever of 39°, severe head pains occurred, and the rash became generalized. The patient was admitted to the county hospital. After a week, a state of coma occurred; vomiting, stroke affecting the extremities on the left side, and then loss of consciousness occurred. The patient was sent to the clinic in Lublin.

At the time of admission, the following was found: the patient was unconscious, and on the skin of the entire body, there was a bright red, spotty rash with ocellar extravasation. The conjunctiva of the eyelids and eyeballs twitched, with cyanosis and swelling of the upper right eyelid. In the internal organs, no deviation from the norm was found. Pulse was 118/min, pulse blood pressure was 110/90 mm Hg. Temperature was 40°. There was rigidity of the neck on the left and in the left fingers, and the nasal-cheek fold was smooth. The upper and lower extremities toward the top fell in a paralysis. Deep reflexes were preserved. Babinski's sign was negative. Stomach reflexes did not succeed in developing. Both eyes were bilaterally unchanged. The action of lumbar pricks indicated a small inflammatory change in the medulla fluid (albumin - 66.0 mg %, polynucleoles 106/3, single nucleoles 16/7).

In additional studies conducted at this time (morphological

composition of the blood, a total urinalysis, O.R., level of urea and sugar in blood serum, liver examination, electrophoresis of albumin in blood serum, O.R., only these deserve attention: leucocytes, 35,200; acidophilic granulocytes, 3%; platey corpuscles, 110,000/cu mm³; time of bleeding and blood coagulation, normal.

EEG study: a pathological record of generalized change of moderate degree. No expression of focal changes was found.

In repeated studies of the feces, no parasite eggs were found. In sections cut from the calf muscle, larva of Trichinella spiralis was not found to be present. In an inhibitory reaction, hemagglutination of antibodies for parasitic encephalitis was not found. A radiological study of the rib cage indicated no change. X-ray of the skull: in the occipital region, visible intervals confirmed traumatic changes. An X-ray of the neck vertebrae was without change.

Taking into consideration the general state of the patient, skin change called to mind a drug reaction and the blood picture was acceptable, that the disease was of toxic-allergic origin. The very high leucocytosis and significant increase in number of acidophilic granulocytes were against this theory.

After five days of treatment, in the course of which the patient received 250 mg of hydrocortisone a day intravenously and sigmoidic temperature began to decrease to 38°, the patient began to react to simple instructions. In the next two weeks, his body temperature returned to normal, the patient regained consciousness, motor activity of the upper and lower extremities returned, and there was found only partial paresis of the spastic type in the extremities on the left side. The skin rash went away. New lumbar pricks indicated only slight pleocytosis, single nucleoles 32/3, albumin 66.0 mg %. Treatment with hydrocortisone was discontinued (the patient received 1,565 mg altogether) and encortone was given orally. On September 3rd, the encortone treatment was ended; the patient still received centrophenoxine and vitamin B complex. He began to walk by himself, but he still sustained a slight paresis in the limbs on his left side. After two days, pruritis of the skin over the whole body appeared; a snotty red rash appeared on the face and limbs, as well as a swelling of the eyelids and face. All drugs were set aside and phenazoline, calcium gluconium, and dexamethasone were given. After a week, the changes in the skin and swelling in the eyelids and face went away. On September 24th, subsequent studies of the feces for parasite eggs indicated the presence of ascarid eggs. The patient stayed on antivermin treatment. On October 3rd, i.e. after two months of treatment, only a small degree of psychological slowness was found. The patient was discharged.

Control studies conducted in the Neurological Clinic on October 17th indicated a small degree of paresis of the right ulnar nerve. Vitamin B₁ and vitamin PP were administered to the patient. After three days, the patient reported to the clinic with complaints of annoying pruritis of the chest skin. A nodular-blistery rash was found on the skin at the side of the rib cage. The rash went away in the course of a few days when treatment was discontinued. Analyzing the course of the disease, it seemed very probable that the symptoms which appeared in the patient could be ascribed to a sensitivity to drugs. In additional investigations of treatment from the

time of injury, it seemed that taking vitamin B₁ preceded every appearance of skin changes.

The patient was admitted to the clinic for a second time. Allergy studies conducted by Dr. K. Żelazowski at the Provincial Dermatology Clinic indicated a conspicuous positive skin reaction to vitamin B₁. With oral administration of two tablets of vitamin B₁ after two hours, pruritis of the rib cage and facial skin appeared in the patient, as well as rubefaction and peeling of the facial skin.

DISCUSSION

Vitamin B₁ is a universal drug for general use without special precaution. The frequency of appearance of sensitivity to this drug is low. But cases are presented in the literature in which signs of allergy or even sudden death were observed with intravenous or intramuscular injection.

Leitner (4) described two cases: a seven-year-old girl, in whom a bronchial asthma attack appeared with eosinophilia, after injection of vitamin B₁; in another case, vitamin B₁ caused insomnia, trembling of the hands, headache, and heart palpitation. Lewis (according to Leitner (4)) told of a case in which, with the injection of vitamin B₁, swelling of the eyelids and lips appeared, nettle marks on the entire body, and collapse with cyanosis. Mille (according to Leitner) described a case of sudden death after intravenous injection of vitamin B₁. In an autopsy in this case, numerous extravasations of the soft meninges were found in both hemispheres of the brain and numerous foci of softening. Barazzone and Lambelet (1) described two cases in which vitamin B₁ injection caused sudden death.

In the case described here, various strong signs appeared with oral administration of vitamin B₁ in small doses. Actually giving vitamin B₁ complex tablets caused a skin rash.

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Zapalenie mózgu w następstwie uczulenia na witaminę B₁

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Schorzenia alergiczne są wyzrzon reakcji zachodzącej pomiędzy antygenem a przeciwciałem, reakcji która powstaje wskutek działania antygenu. Rodzaj i stopień nasilenia tej reakcji zależy od odczynowości ustroju, która może być różna; jeśli jest więkza od przeciętnej może dać objawy alergiczne. Znamienne jest to, że przy wysokiej odczynowości alergicznej ustroju także nieswoiste bodźce mogą wywołać ten sam odczyn alergiczny. W wyniku tego obraz schorzeń alergicznych bywa różny.

Schorzenia alergiczne układu nerwowego cechuje również znamienność dla alergii wielopostaciowość objawów i przebiegu. Odczyn alergiczny może dotyczyć tkanki układu ośrodkowego, nerwów obwodowych i opon mózgowo-rdzeniowych (2, 3).

Przedstawiony przez nas przypadek wydaje się interesujący ze względu na etiologię, wyjątkowo ciężki przebieg oraz zmiany skórne przypominające osutkę odrową.

Opis przypadku

S. J. 25-letni, (hist. chor. nr 759 69) z zawodu kierowca, skierowany do kliniki 6.VIII.1969 ze szpitala powiatowego z podejrzeniem zapalenia mózgu i opon mózgowych w przebiegu odry.

Chory przed miesiącem doznał urazu głowy z utratą przytomności w wypadku drogowym. Z powodu trwających się bólów głowy był leczony 7 dni w szpitalu, a następnie w Wojewódzkiej Poradni Neurologicznej 26.VII.1969 przeżył śle. wystąpiły bóle w jamie brzusznej i żywo-czerwona wysypka w skórze brzucha i klatki piersiowej. Po 2 dniach chory zagorączkował do 38°C, wystąpiły silne bóle głowy, omdlała i utracił przytomność. Po tygodniu wystąpił stan zamroczenia, wyndoty, porażenie kończyn i na stronie lewej, a następnie utrata przytomności. Chorego przesłano do kliniki w Lublinie.

W chwili przyjęcia stwierdzono: chory nieprzytomny, w skórze całego ciała żywo-czerwona osutka składająca się z punkcikowatymi wybroczynami. Spojówki powłok błon śluzowych i błon łąkowych ocznych natrząknięte, obrzęk i zaczerwienienie błony śluzowej górnej prawej. W narządach wewnętrznych oddechowa

porny nie stwierdzono. Tetno 118/min, ciśn. krwi tętnicze 110/90 mm Hg. Ciepłota ciała 40°. Sztwność karku na 1 palec. Lwy fałd nosowo-policzkowy wyglądający. Konczyny górne i dolne umieszczone ku gorze opadają bezwładnie. Odruchy głębokie zachowane. Objaw Babinskigo ujemny. Odruchy brzusznych nie udaje się wywołać. Dno oczu obustronnie bez zmian. Wykonane nakłucie lędźwiowe wykazało niewielkie zmiany zapalne w płynie mózgowo-rdzeniowym (białko — 66,0 mg%, 106,3 wielojąd. 16,3 jednojąd.).

W badaniach dodatkowych wykonanych w tym czasie (skład morfologiczny krwi, badanie ogólne moczu, OB, poziom mocznika i cukru w surowicy krwi, próby wątrobowe, elektroforeza białek surowicy krwi, WR) na uwagę zasługują jedynie: leukocytoza 35 200; granulocytów kwasochłonnych 36%; Krwinek płytkowych 110 000/mm³, czas krwawienia i krzepnięcia krwi w normie.

Badanie Eeg: zapis patologiczny o zmianach uogólnionych średniego stopnia. Wyraźnych zmian ogniskowych nie stwierdzono.

W kilkakrotnym badaniu kału, jaj pasożytów nie znaleziono. W wycinkach z mięśni łydki nie stwierdzono obecności larw *trichinella spiralis*. W odczynie zahamowania hemaglutynacji przeciwciał dla kleszczowego zapalenia mózgu i pokrewnych nie stwierdzono. Badanie radiologiczne klatki piersiowej nie wykazało zmian. Rtg czaszki: w okolicy kości potylicznej widoczne przejaśnienia przemawiające za zmianami pourazowymi. Rtg kręgosłupa szyjnego bez zmian.

Biorąc pod uwagę ogólny stan chorego, zmiany skórne przypominające osutkę odrów oraz obraz krwi przyjęto, że schorzenie jest pochodzenia toksyczno-alergicznego. Przeciw odrze przemawiała bardzo wysoka leukocytoza i znaczne podwyższenie liczby granulocytów kwasochłonnych.

Po 5 dobach leczenia, w czasie których chory otrzymywał po 230 mg hydrokortyzonu dożylnie na dobę i sigma-mycynę ciepłota ciała obniżyła się do 38°, chory zaczął reagować na proste polecenia. W ciągu następnych 2 tygodni ciepłota ciała powróciła do normy, chory odzyskał przytomność, powróciła ruchomość kończyn górnych i dolnych, stwierdzono tylko częściowy niedowład typu spastycznego kończyn po stronie lewej. Osutka w skórę ustąpiła. Ponowne nakłucie lędźwiowe wykazało tylko niewielką płecystą jednojądrzastą 32,3, białko — 66,0 mg%. Zakończono leczenie hydrokortyzonem (chory otrzymał łącznie 1 565 mg) i podawano doustnie enkorton. W dniu 3.IX. kurację enkortonem zakończono, chory otrzymywał jeszcze centofeneksin oraz witaminę B comp. Zaczął chodzić o własnych siłach, utrzymywał się jeszcze lekki niedowład kończyn po stronie lewej. Po 2 dniach wystąpił świąd skóry całego ciała, na twarzy i na kończynach pojawiła się plamisto-czerwona osutka, wystąpił obrzęk powiek i twarzy. Odstawiono wszystkie leki i podano fenazolinę, calcium gluconicum oraz deksametazon. Po tygodniu zmiany skórne, obrzęk powiek i twarzy ustąpiły. 24.IX. kolejne badanie kału na jaja pasożytów wykazało ciemność jaja glisty ludzkiej. Chory przebieg kurację antyhelmintyczną. 3.IX. tj. po 2-miesięcznym leczeniu stwierdzono tylko niewielkiego stopnia spowolnienie psychiczne. Chory został wypisany do domu.

Badanie kontrolne przeprowadzone w Przychodni Neurologicznej w dniu 1.IX. wykazało niewielkiego stopnia niedowład prawego narułu łokciowego. Przepisano choremu wit. B₁ i wit. B₁₂. Po 2 dniach chory zgłosił się do przychodni ze zmianami na dotychczasowy świąd skóry klatki piersiowej. Stwierdzono osutkę grudkowo-pęcherzową w skórę łeczną oraz klatki piersiowej. Po odstąpieniu leków chory ustąpił w przebiegu kilku dni. Kolejne badanie przychodni wykazało się najbardziej powolnym, nie objawiając się w postaci u choro, nie było przypisać nadwrażliwości na leki. Po dalszym przywróceniu leczenia od chwili czasu choroby się, ze zatykanie witamin B₁ i B₁₂ powodowało wystąpienie zmian skórnych.

Chorego przyjęto do kliniki po raz drugi. Badanie alergologiczne przeprowadzone przez dr. med. K. Zelażowskiego w Wojewódzkiej Przychodni Dermatologicznej wykazało wyraźne dodatni odczyn skóry na witaminę B₁. Po doustnym podaniu 2 tabletek wit. B₁ wystąpił u chorego każdziarstwo po 2 godzinach świąd skóry i ławki piersiowej i twarzy oraz zaczerwienienie i buszczenie się skóry twarzy.

Omówienie

Witamina B₁ jest lekiem powszechnie stosowanym na ogół bez specjalnej ostrożności. Częstość występowania nadwrażliwości na ten lek jest stosunkowo niewielka. Podano jednak w piśmiennictwie przypadki, w których po wstrzyknięciu dożylnym lub doustnym obserwowano objawy uczulenia a nawet nagłą śmierć.

Leitner (4) opisał 2 przypadki: dziecka 7-letniego u którego po wstrzyknięciu wit. B₁ wystąpił napad dychawicy oskrzelowej z czynofilią; w drugim przypadku wit. B₁ wywołała bezsenność, drżenie rąk, ból głowy i kołatanie serca. Lews (wg Leitnera 4) donosi o przypadku, w którym po wstrzyknięciu wit. B₁ wystąpił obrzęk powiek i warg, pokrzywka na całym ciele i zapas z sinicą. Mille (wg Leitnera 4) opisał przypadek nagłej śmierci po wstrzyknięciu dożylnym wit. B₁. Badaniem sekcijnym w tym przypadku stwierdzono liczne wybroczyny w oponach mózgowych nad obu półkulami mózgu i liczne ogniska rozrzętnia. Barazzone i Lambelet (1) opisują 2 przypadki, w których wstrzyknięcie wit. B₁ spowodowało nagłą śmierć.

W opisanym przypadku ciężkie objawy wystąpiły doustnym stosowaniu witaminy B₁ w niewielkich dawkach; nawet podanie tabletek witaminy B₁ spowodowało odczyn skórny.

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A case of allergic encephalitis is described in a 25-year-old man following oral administration of vitamin B₁. The course of the disease was extremely severe and eruptions were seen covering the whole body, it resembled the measles rash but was followed by pruritis.

Allergologic investigations showed a statistically significant skin reaction to vitamin B₁. Every oral administration of vitamin B₁ tablets caused itching of the chest skin, erythema and scaling of the chest skin.

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14: 1444-1448 (1966)]

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Hydrolytic Cleavage of Thiamine in Mammalian Animals

The metabolic fate of thiamine has been studied in various species of animals, but relatively little is known of how it is metabolized in the mammalian body. Verrett, *et al.*¹⁾ reported that oral and parenteral routes of administration did not make great differences in the metabolic pattern of ³⁵S-thiamine in rabbits.

The results of the present study showed that the metabolic pattern of ³⁵S-thiamine in rats was remarkably different between oral and parenteral routes of administration. Female Wistar rats weighing 150-200 g. were used. They were housed in cages constructed to permit the separate collection of urine and feces. ³⁵S-Thiamine with a specific activity of 25 μ Ci./mg., was prepared from C¹⁴S₂ according to the procedure of Matsukawa.²⁾ A dose of 0.2 mg. of ³⁵S-thiamine was administered orally and intravenously. Twenty four hour urine specimens were collected in glass bottles. For separation of the urinary metabolites, paper chromatography was employed. A part of the pooled urine was spotted on Toyo filter paper No. 51 and developed with *n*-butanol-acetic acid-water (4:1:5, v/v). Radioactive scanning of paper chromatograms was accomplished by dividing the chromatograms in 10 mm. segments, extracting each segment with distilled water and counting ³⁵S radioactivity of each extract in a windowless gas flow counter. No attempt was made to correct for recovery of the radioactivity from chromatograms and sample absorption in extracts. Scintillation counting was used to determine the recovery of the administered radioactivity from the urine. The percentages of ³⁵S radioactivity in urine represented by radioactive metabolites of ³⁵S thiamine are indicated in Table I.

- 1) M.I. Verrett, I.R. Cerecedo: Proc. Soc. Exp. Med. Biol., **98**, 509 (1958).
- 2) T. Matsukawa, T. Iwazu: Yakugaku Zasshi, **70**, 28 (1950).

From data in Table I, it is evident that the metabolic pattern of ^{35}S -thiamine is considerably different between oral and parenteral routes of administration. The Rf value of authentic ^{35}S -thiamine is 0.24, and hence most of the radioactivity in peak no. 2 are considered to be unchanged ^{35}S -thiamine. When ^{35}S -thiamine was injected intravenously, 65.5% of the excreted radioactivity occurred in the area corresponding to thiamine, as shown in Table I. This datum is in agreement with the result of Iacono *et al.*³⁾ that approximately 60% of the excreted radioactivity occurred in the thiamine area when ^{14}C -thiamine was injected intraperitoneally into rats. On the other hand, when ^{35}S -thiamine was administered orally, only 6.5% of the excreted radioactivity was observed in the thiamine area, and 81.0% was found in the area corresponding to Rf value of 0.80~0.90. Since the Rf value of the unknown compound was high, it was assumed that this compound might have high lipid-solubility.

When the urine of rats receiving 10 mg of ^{35}S -thiamine was extracted with chloroform, it was found that more than 70% of the excreted radioactivity was transferred into chloroform layer. The extract did not fluoresce when treated with potassium ferricyanide, which converts thiamine and thiamine derivatives into their corresponding thiochrome derivatives, whereas it colored when treated with Dragendorff's reagent. These facts led to an assumption that the unknown compound in peak no. 6 might be thiazole moiety of thiamine.

Thiazole moiety of thiamine has been reported to occur in rat and rabbit urine by several groups of workers.^{1,4-6)} Recently, Ogawa⁶⁾ described that 4-methyl-5 β -hydroxyethylthiazole (HT) could be identified in rabbit urine by paper chromatography. Therefore, HT was prepared from thiamine according to the procedure of Matsukawa,⁷⁾ and chromatographed in *n*-butanol-acetic acid-water (4:1:5, v/v), along with the chloroform extract of rat urine. As a result, its Rf value was identical to that of the unknown compound. The range of Rf values obtained with the unknown compound and with HT in 3 solvent systems is shown in Table II. To ascertain the unknown compound from urine to be HT, isotope dilution method was applied.

The experimental details are as follows: The pooled urine was concentrated *in vacuo* and then extracted with alcohol. To the alcohol solution was added 100 mg. of non-labeled HT and the solvent was evaporated. The residue was extracted with dry chloroform and the chloroform layer was extracted with *N*/10 hydrochloric acid. The aqueous solution was adjusted to pH 9.0 with *N* aqueous ammonia and then extracted with chloroform. The chloroform layer was dried over sodium sulfate and the solvent was evaporated. The residue was dissolved in 2 ml. of alcohol, and to alcohol solution was added the alcohol solution containing 300 mg. of picric acid. The crystallized picrate was recrystallized from alcohol and then from hot water. Specific activity of each picrate was determined. As the results, specific activities of first, second and third picrates were 9400 c.p.m./mg., 9200 c.p.m./mg., and 9150 c.p.m./mg., respectively. The slight decrease in specific activity was thought to be due to the presence of non-radioactive other picrate. From these data above described, it is evident that ^{35}S HT is excreted in rat urine after oral administration of ^{35}S -thiamine. This compound, which is known to be a product of the action of a specific bacterial thiaminase,⁸⁾ would be generated by hydrolytic cleavage of the covalent bond between the methylene bridge and thiazole

3) J.M. Iacono, B.C. Johnson: J. Am. Chem. Soc., **79**, 6321 (1957).

4) P.T. McCarthy, L.R. Cerecedo, E.V. Brown: J. Biol. Chem., **209**, 611 (1954).

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ring. The occurrence of the pyrimidine moiety of thiamine in urine has been reported. Kawasaki, *et al.*⁹ isolated 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP) from human urine after giving large doses of thiamine to test subjects. Neal, *et al.*¹⁰ reported that 2-methyl-4-amino-5-pyrimidinecarboxylic acid, an oxidation product of HMP, was found in the urine of rats receiving ¹⁴C-thiamine. The marked difference in metabolic pattern of ³⁵S-thiamine brought about by different routes of administration suggests that formation of HT from thiamine occurs in the gut. A few percent of ³⁵S-HT was detected in rat urine even after parenteral administration of ³⁵S-thiamine, as shown in Table I. This may be explained by taking account of the fact

TABLE I. Percentage of ³⁵S Radioactivity in Rat Urine Present as Radioactive Metabolites after Oral and Parenteral Administration of ³⁵S-Thiamine

Peak No.	Rf value	Corresponding compound	Oral admin. (6) (%)	Intravenous injection (2) (%)
1	0.0 ~ 0.15	thiamine phosphates ^{a)}	1.5	25.5
2	0.20 ~ 0.30	thiamine	6.5	65.5
3	0.35 ~ 0.45	thiochrome ^{b)}	0.5	1.5
4	0.50 ~ 0.60		0.5	1.0
5	0.65 ~ 0.75		7.0	2.0
6	0.80 ~ 0.90	HT	84.0	4.5

Values in parentheses indicate number of experiments.

a) Rf values of authentic thiamine monophosphate and thiamine diphosphate are 0.10 and 0.06, respectively.

b) Rf value of thiochrome is 0.40.

that the injected thiamine is secreted into the gut lumen.¹¹

Since it is known that bacterial thiaminase occurs in the gut, there is a possibility that HT may be produced microbiologically.

However, Neal, *et al.*¹⁰ described that this possibility would have to be excluded, since even in the urine of germ-free rats the presence of 2-methyl-4-amino-5-pyrimidinecarboxylic acid was demonstrated. The results of chromatographic analyses of the radioactivity in portal venous blood, intestinal wall, liver and kidney one hour after oral administration of ³⁵S-thiamine are shown in Table III. The percentage of

TABLE II. Range of Rf Values for the Unknown Compound and for 4-Methyl-5β-hydroxyethylthiazole (HT) in Various Systems

Solvent system	Rf values	
	Unknown compound	HT
n-butanol-acetic acid-water 1:1:5, v/v	0.83~0.88	0.83~0.85
n-propanol-water-acetate buffer pH 5.65:20:15, v/v	0.90~0.94	0.92
n-butanol saturated with water	0.33~0.36	0.35

³⁵S-HT in portal venous blood was the highest and that in intestinal wall was the lowest. This also indicates that only in the gut HT is formed from thiamine and suggests that the transport of HT from intestinal wall into portal blood takes place extremely rapidly as compared to that of thiamine.

9. T. Kawasaki, K. Okada: *Vitamins*, **13**, 351 (1961).

10. R.A. Neal, W.N. Fennell: *J. Nutrition*, **83**, 351 (1964).

11. B. Gussmann, H.A. Kutz: *Biochem. Z.*, **334**, 245 (1961).

TABLE II. Percentage of ^{35}S Radioactivity in Blood, Intestinal Wall, Liver and Kidney Present as Radioactive Metabolites One Hour after Oral Administration of ^{35}S -Thiamine

Rf value	Portal blood (0.18 g/ml.) (%)	Intestinal wall (2.2 g/g) (%)	Liver (0.28 g/g) (%)	Kidney (0.59 g/g) (%)
0.0 ~ 0.15	4.0	16.0	18.5	2.5
0.20 ~ 0.30 (thiamine)	5.0	56.5	12.0	3.0
0.35 ~ 0.45	2.0	1.5	1.0	1.0
0.50 ~ 0.60	1.0	0	0	0
0.65 ~ 0.75	6.0	0	26.0	14.0
0.80 ~ 0.90 (HT)	32.0	26.0	42.5	79.5

Values in parentheses indicate percent recovery of administered radioactivity. Percentages in four columns indicate the mean values of two animals.

To know whether the hydrolytic cleavage of thiamine occurs in other mammalian animals, the similar experiments were made with adult guinea pig, mouse and rabbit. As shown in Table IV, it was found that ^{35}S HT was excreted in the urine of all of the animals studied. However, percentages of ^{35}S HT in the urine of these animals were smaller than that in rat urine. The excretion of ^{35}S radioactivity and unchanged ^{35}S -thiamine in urine 24 hours after oral administration of ^{35}S thiamine is shown in Table V. Percent recovery of the administered radioactivity was the highest in rat

TABLE IV. Percentage of ^{35}S Radioactivity in Urine of Mammalian Animals Present as Radioactive Metabolites after Oral Administration of ^{35}S Thiamine

Rf value	Corresponding compound	Rabbit (2) (%)	Mouse (2) (%)	Guinea pig (4) (%)
0.0 ~ 0.15	thiamine phosphates	9.0	8.3	20.2
0.20 ~ 0.30	thiamine	40.7	40.5	41.2
0.35 ~ 0.45	thiochrome	3.5	1.7	4.6
0.50 ~ 0.60		3.0	3.0	6.0
0.65 ~ 0.75		6.3	18.7	3.3
0.80 ~ 0.90	HT	37.5	27.8	22.7

Values in parentheses indicate number of experiments.

TABLE V. Excretion of ^{35}S Radioactivity and ^{35}S Thiamine in Urine of Mammalian Animals 24 Hours after Oral Administration of ^{35}S Thiamine

Animal	No. of exp.	Admin. dose (μg /animal)	Recovery of ^{35}S (% of admin. ^{35}S)	Recovery of ^{35}S thiamine (% of admin. ^{35}S)
Rat	6	200	58.0	1.77
Guinea pig	4	200	9.8	1.04
Rabbit	2	2000	30.5	1.42
Mouse	2	50	29.3	11.35

a) Values in this column were obtained by multiplying the percentage of the radioactivity in the area of Rf value 0.20~0.30, which was shown in Table II and IV by the percentage of total radioactivity in the urine, which was shown in Table I.

and the lowest in guinea pig. When one compares the percent recovery of the total radioactivity and unchanged ^{35}S thiamine in rat with that in guinea pig receiving the same dose of ^{35}S -thiamine, one finds that the total radioactivity excreted in rat urine is six times as high as that in guinea pig urine although the amount of unchanged

^{35}S thiamine in rat urine is similar to that in guinea pig urine. This is apparently due to the fact that more than 80% of the radioactivity excreted in rat urine is present as ^{35}S -HT whereas in guinea pig urine only about 20% is ^{35}S HT. This, in turn, indicates that the hydrolytic cleavage of thiamine occurs much more actively in rat than in guinea pig. It should be noted that only about 4% of the administered ^{35}S -thiamine was excreted as unchanged ^{35}S -thiamine in rat and guinea pig urine within 24 hours when a dose of 200 μg . of ^{35}S -thiamine was orally administered.

This data approximately agree with the report of Fukutomi¹²⁾ that 5% of the administered thiamine was excreted in rat urine within 5 days when 500 μg . of thiamine was orally administered for 4 days. To know whether or not bacterial thiaminase takes part in hydrolytic cleavage of thiamine in rat, further experiments are now in progress.

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To the Editor:—The use of thiamine hydrochloride, through both lay and medical channels, has reached large proportions. With inferential evidence only regarding human requirements for the maintenance of good health, and without any published evidence of toxicity, the tendency has been toward an intake well above established deficiency levels. Williams and his associates (Williams, R. D.; Mason, H. L.; Wilder, R. M., and Smith, B. F.: Observations on Induced Thiamine Deficiency in Man, *Arch. Int. Med.* 66:785 [Oct.] 1940) showed that definite deficiency symptoms would develop in otherwise normal adults kept on a daily intake of 0.15 mg. for several months, while it is generally presumed that 2 to 3 mg. daily will cover the normal adult needs in health. Little evidence has been presented, however, to show just how much the daily intake need be increased in patients suffering from long-standing deficiency. Here again the tendency has been toward the use of large doses, with a daily intake many times the normal requirement. Such therapy can reasonably be based only on a clearly established nontoxicity and on evidence that such excessive dosage carries therapeutic benefits not obtained by dosages down nearer the normal requirement. Neither of these bases has yet been satisfactorily established by published data.

In August 1940 I observed an onset of definite toxicity in a Cincinnati woman aged 47 who had been taking 10 Gm. of thiamine hydrochloride daily for two and one-half weeks. The symptoms resembled those of overdosage with thyroid extract—headache, increased irritability, insomnia, rapid pulse, weakness and trembling—and cleared up within two days after administration of thiamine hydrochloride was discontinued. After one week's rest the patient began taking 5 mg. of thiamine hydrochloride daily and after four and one-half weeks at this intake level the same toxic syndrome recurred. Prompt relief again followed cessation of the intake of thiamine hydrochloride.

During a recent visit to Panama, I observed other patients with thiamine toxicity. Liberal doses (20 to 40 mg. of thiamine hydrochloride daily) are often prescribed by physicians in tropical climates in an effort to overcome the physical let-down which so commonly afflicts persons migrating there from cooler regions. One young woman, receiving an average of 17 mg. daily, was excreting 12 mg. daily in her urine and passing stools smelling strongly of thiamine hydrochloride. She was showing symptoms similar to those of thyroid hyperactivity, with fine and coarse muscle tremor, rapid pulse and noticeable nervous hyperirritability. Several other similar cases of toxicity were reported to me after attention had been drawn to the type of toxic symptoms one might expect from overdosage. Cessation of the administration of thiamine hydrochloride was followed in each such case by prompt subsidence of the hyperthyroid-like symptoms. A detailed report of a series of these instances of overdosage will soon be offered for publication by Dr. F. A. Raymond of the Panama Hospital.

Just why no reports of thiamine toxicity have appeared in medical literature is difficult to understand, for the vitamin has been widely used at daily intake levels of 10 to 50 mg. in treatment of deficiency states. Prevalence of toxic reactions in the tropics (or in Cincinnati's August heat) may be related to more widespread multiple deficiencies for the B vitamins at the high environmental temperatures. In cases of multiple deficiency it has been shown that unfavorable results may attend administration of a single one of the lacking elements (Morgan, Agnes Fay: The Effect of Imbalance in the Filtrate Fraction of the Vitamin B Complex in Dogs, *Science* 88:261 [March 14] 1941). Studies on rats in my laboratory (Mills, C. A.: Environmental Temperatures and Thiamine Require-

ments, *Am. J. Physiol.*, to be published) have shown thiamine (and pantothenic acid) requirements per gram of food to be decidedly higher for animals kept at 90 F. than at 65 F. environmental temperature level. It may perhaps be the greater prevalence of multiple deficiency in regions of tropical heat that has led to the unfavorable results of high thiamine dosage in Panama. Lower requirement per gram of food in temperate zone coolness may have prevented similar evidences of unfavorable reactions from appearing at more northern latitudes.

Recognition of symptoms of overdosage at once necessitates more adequate information as to actual thiamine needs under various conditions. Such information can be obtained only by blood level and excretion rate studies. Addition of thiamine hydrochloride to bread and other commonly used foods, without control over the intake level, also carries a potentiality of harm. This is particularly true in those stimulating middle temperate regions where hyperthyroidism and other forms of metabolic disturbance are already prone to occur. The medical profession should recognize the need for greater conservatism in the use of this important vitamin, and its distribution through both nonmedical and medical channels should be placed on a more scientific and controlled basis.

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VITAMINS AS PHARMACOLOGICAL AGENTS

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A discussion of vitamins as pharmacological agents appears at first to be a rather uninteresting undertaking, since in comparison with other potent remedies these substances fail to produce conspicuous pharmacodynamic effects. Indeed, the range between the therapeutic and the lethal dose is so wide, that one might feel justified to place vitamins in the same category as salt, sugar, or other basic food elements which are essential from the nutritional viewpoint, but practically inert in the pharmacological sense. However, such an opinion would be erroneous since vitamins are pharmacologically quite active,

provided they are investigated in animals suffering from a specific vitamin deficiency.

The fact that a drug, in order to exhibit its pharmacodynamic effect, has to be tested under certain pathological conditions, is not unusual. Antipyretics, spasmolytics and analgesics may be cited as other examples. However, while the pathological condition necessary for demonstrating the effect of these drugs may be produced in a variety of ways, a vitamin affects only those conditions which result from a deficiency of that particular agent; in this respect, vitamins resemble in their therapeutic specificity the immune sera

or antitoxins. Failure to recognize this accounts not only for a large number of ill-founded therapeutic claims, but also for many of the disappointments of vitamin therapy and the skepticism originating therefrom.

It can readily be seen, that a discussion of vitamins as pharmacological agents must distinguish between their effect in the normal and in the vitamin deficient animal. In normal animals their action is rather unspecific and pharmacodynamic effects are produced only by doses several thousand times larger than those needed for a therapeutic effect (table 1).

The only vitamin which produces a distinct pharmacologic effect even in therapeutic doses, is nicotinic acid. Its administration is followed in humans by a transitory peripheral vasodilatation. However, this effect cannot be ascribed to its nature as a vitamin, since it fails to occur on the administration of nicotinamide, which is equally effective as a vitamin.

TABLE 1

Lethal doses of B vitamins as compared with their daily requirement

	SPECIES	DAILY RE- QUIREMENT	L.D. 50 ORAL	RATIO
Thiamin	mouse	3 γ	100 mgm.	30,000
Riboflavin	mouse	8 γ	>7 mgm.	>8,000
Nicotinic acid	dog	0.25 mgm. ¹	>3 grams ²	>1,000
Pyridoxine	rat	10 γ	1 gram ²	100,000
Pantothenic acid	rat	100 γ	>3 grams ²	>30,000

¹ Per kilogram body weight.

² Per rat (300 grams body weight).

With therapeutic margins of from 1:1000 upward, the danger of acute toxicity is obviously of very minor concern to practical vitamin therapy. The question of chronic toxicity deserves more attention since vitamins, due to their nature as nutritional factors, are likely to be taken over a prolonged period of time and without supervision by a physician. Fortunately, all information presently available indicates that the danger of chronic hypervitaminosis is practically non-existent. Daily feeding of up to several hundred times the maintenance doses of thiamin, riboflavin, pyridoxine, nicotinic acid and pantothenic acid over the entire life span of rats fails to produce toxic effects. However, since pathologic changes resulting from prolonged administration of large doses of vitamins may become manifest only in the second generation, toxicity experiments should be extended beyond the first generation. Thus, Perla (1) has shown that in rats prolonged administration of thiamin causes in the third generation loss of maternal instinct and interferes with lactation; Suro (2) has made similar observations and we obtained such findings in an experiment extended over 4 generations.

According to Perla (3), this toxicity is due to a relative lack of manganese in the diet and can be prevented by addition of traces of this element. Thus, ordinarily innocuous doses of vitamins may become harmful under certain conditions. Unfortunately, not much experimental data is as yet available to elaborate on this point. V. A. Drill (4) reported, that hyperthyroid female rats, which had lost weight and were still receiving a thyroid preparation, regained their weight when both thiamin and vitamin B complex were administered; and Suro and Smith (5) found that in such animals the weight loss could be retarded by administration of a potent vitamin B concentrate. In a later paper, Suro (6) and Buchanan (6) state that pure thiamin in daily doses of 30 to 100 micrograms counteracts the toxic effects of 0.2 mgm. thyroxine daily. In our laboratory we have

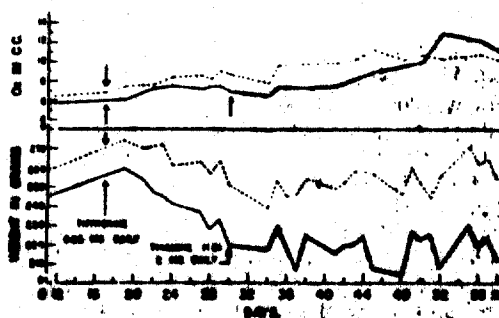


Fig. 1. Effect of thiamine on weight and O_2 consumption of hyperthyroid rats. Curves represent the averages of a group of 4 rats. All animals received 0.25 mgm. thyroxine and those represented in the lower curve received 2 mgm. thiamin hydrochloride at the point indicated.

investigated the influence of hyperthyroidism on the toxicity of very large doses of thiamin. Rats were given daily doses of thyroxine and simultaneously, or after establishment of a hyperthyroid state, large amounts of thiamin were administered. In addition to daily determination of the oxygen consumption and body weight frequent records were taken of the heart and respiratory rate and the body temperature. Although more than 200 rats were used in these experiments, the normal and hyperthyroidic rats did not differ significantly in their sensitivity to thiamin (fig. 1).

A question of practical importance is whether an imbalance in a combination of vitamins may lead to toxic effects which are not observed, if the vitamins are given in their naturally occurring mutual relationship. This problem has attained particular prominence since the enrichment of foods with selected members of the vitamin B

complex has become an established practice, although even those investigators who believe in the possibility of an increased vitamin toxicity due to imbalance have always emphasized, that this danger does not exist for the relatively minute quantities of vitamins incorporated into flour, bread or other foodstuffs. The only published experimental data indicating an adverse effect of vitamin imbalance are those of A. F. Morgan (7), who showed that dogs suffering from a combined nicotinic acid and pantothenic acid deficiency lived longer than those on nicotinic acid or pantothenic acid deficiency alone.

In our laboratory the effect of pantothenic acid or riboflavin was studied in adult dogs during a combined pantothenic acid-riboflavin deficiency (table 2). No increase in toxicity of either vitamin was found in these conditions. Whether the dogs were on the combined deficiency or only on riboflavin deficiency, the same length of time

adverse effect of a vitamin imbalance because they may merely indicate that some deficiencies require more time for development than others, time which is provided in these experiments through the cure of that deficiency which occurred first.

In a series of experiments undertaken in our laboratory, the effect of prolonged administration of large doses of single vitamins of the B group on the course of multiple deficiencies was investigated. There was no indication of an increased toxicity (14). Similarly, we have been unable to demonstrate a greater toxicity of thiamin in animals suffering from riboflavin deficiency (table 3).

As with other medicinal agents, the question of optimal dosage, most effective spacing and preferred way of administration deserves careful consideration.

It appears advisable to distinguish between a maintenance dose, sufficient to provide an adequate supply of the vitamin in a non-depleted organism and a curative dose, necessary to restore

TABLE 2
Progress of depletion symptoms in riboflavin and riboflavin-pantothenic acid deficient dogs

Dog No.	Deficiency	Collapse after weeks	RIBOFLAVIN EXCRETION IN γ /24 HOUR				
			1st week	15th week	22nd week	28th week	29th week
108	Riboflavin	23	77	15	6		
115	Riboflavin	26	30	15	17	8	
105	Riboflavin and Pantothenic Acid	35	176	28	27	26	11
113	Riboflavin and Pantothenic Acid	22	87	13	8		

was required to produce collapse and the pre-collapse level of riboflavin in the urine was essentially the same in both groups.

It has been reported that during a multiple deficiency correction of only one component tends to aggravate the symptoms of the other deficiencies. Sydenstricker (8), Spies and others described the development of nicotinic acid and riboflavin deficiency symptoms in sub-acute cases of pellagra treated only with thiamin. György (9), Chick (10) and Harris (11) have shown that in rats maintained on diets deficient in both riboflavin and vitamin B₆, the dermatitis characteristic of the lack of the latter does not become manifest until riboflavin is supplied. Daft and Sabrell (12), and Lepkevsky, Jukes and Krause (13) have made similar observations on the appearance of pantothenic acid deficiency lesions in rats deficient both in pantothenic acid and pyridoxine. However, such observations must be interpreted as evidence for an

TABLE 3
Acute toxicity of thiamine in normal and riboflavin deficient rats

MODE OF ADMINISTRATION	NORMAL RATS		RIBOFLAVIN DEFICIENT RATS	
	L.D. 0	L.D. 50	L.D. 0	L.D. 50
	mgm./kgm.	mgm./kgm.	mgm./kgm.	mgm./kgm.
S.C.	500	1400	>750	1500
P.O.	6000	9500	8000	9000

to normal a vitamin depleted animal. Obviously, the curative dose is usually much larger than the maintenance dose, making it advisable to administer the vitamin in its pure form and, quite frequently, by parenteral injection.

Contrasted to this strictly medicinal use is the equally essential use of vitamins for the maintenance of dietary balance. The doses required for this purpose are much smaller than those needed for therapy and can be supplied through a carefully selected diet or specially prepared and enriched foodstuffs.

Since there is practically no danger of overdosing with any of the vitamins, the principal points of interest are those related to absorption, storage and excretion as they have a direct bearing on the mode of administration. The various groups of vitamins differ greatly in these properties. Generally speaking, the fat soluble vitamins A and D are stored to a greater extent than the water soluble vitamins B and C. This influences not only the rapidity with which deficiencies may develop, but also the efficiency of different modes of administration.

As with other medicinal agents, the availability of a vitamin to the organism is determined by the extent to which it may be stored, absorbed, destroyed and excreted. Since all of these factors are closely interrelated, conclusions which are based on only one of them must be drawn with great reservation. Thus, the difference between the dose administered and excreted is not necessarily an indication of the basic requirement. For example, of a dose of 100 micrograms of pantothenic acid given to a rat, 7 micrograms were excreted. However, when 250 micrograms were given only 67 were excreted and with 3000 micrograms only 600 micrograms could be found in the urine. On the basis of these findings, the requirement would appear to be 93, 183 and 2400 micrograms respectively, while actually it is about 100 micrograms. The difference in the foregoing findings must be attributed either to storage, non-absorption or destruction; as a rule each of these factors plays a rôle.

TABLE 4
Oral and intravenous toxicity of B vitamins in rats

	INTRAVENOUS	PERORAL
	gm./kgm.	gm./kgm.
Thiamin	0.170	9.5
Riboflavin	0.540	more than 10
Nicotinamide	2.2	3.5
Pyridoxine	0.657*	6.5
Pantothenic acid	0.630	more than 10

* Weigand, Kehler and Chen, Proc. Soc. Exp. Biol. & Med., 44, 147, 1940.

Vitamins being essential food elements, they can obviously be effectively administered by mouth. However, under certain pathological conditions, such as severe gastro-intestinal disturbances, their absorption may be greatly delayed or even completely absent and in these cases parenteral administration is indicated.

Administration of vitamins by other than the peroral route influences the relative toxicity as well as the efficacy. As with most other drugs, the acute intravenous toxicity is generally considerably greater than the subcutaneous, and in turn, the peroral (table 4). Furthermore, the intravenous toxicity depends much upon the rate of injection (table 5). Whether the intravenous or subcutaneous administration of a vitamin is therapeutically more effective than its peroral administration depends upon several factors of which absorption from the gastrointestinal tract is only one. Intravenous injection produces not only (though temporarily) a much higher concentration of the vitamin in the blood, but probably also in the tissues. Thus, intravenous injection

of 4 mgm. per kgm. of riboflavin to dogs in the form of its sodium salt raises the blood level 15 to 20 times above that obtained by feeding an excess dose by mouth; and the riboflavin content in the liver of rats increased from 35 micrograms per gram to 100 micrograms per gram after a dose of 500 mgm. per kgm.

The fact that parenteral administration can raise the concentration of a vitamin in the blood and tissues far above that obtainable by feeding of even the largest doses may explain certain therapeutic successes obtained by clinicians through parenteral administration of relatively enormous doses of vitamins. Furthermore, with

TABLE 5
Influence of rate of injection on intravenous thiamine toxicity

DOSE	NO. OF ANIMALS	VOLUME INJECTED	CONCENTRATION OF SOLUTION	RATE OF INJECTION	NO. DEAD
mgm./kgm.		c.c.	per cent	cc./min.	
50	10	0.25	0.72	0.1	1
50	10	0.25	0.72	0.3	5
50	10	0.25	0.72	0.4	7

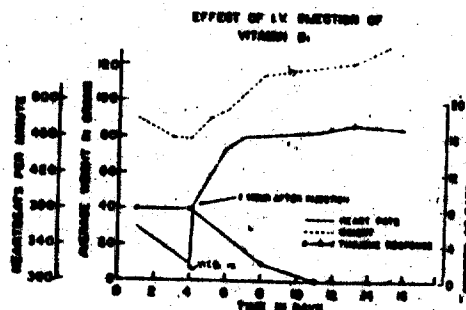


Fig. 2. Effect of intravenous injection of vitamin B₁.

this mode of administration the pharmacodynamic effect is likely to take place with great rapidity. Thus, the effect of thiamin on bradycardia due to deficiency of this vitamin can be observed within less than 1 hour (fig. 2). Still more dramatic is the life-saving effect of intravenously injected vitamins to animals in collapse from pantothenic acid or riboflavin deficiency or to beriberi patients suffering from acute cardiac decompensation. However, even when given by mouth, the therapeutic effect of most vitamins takes place very rapidly, provided the absorption from the gastro-intestinal tract is still normal.

While certain therapeutic effects may be more readily achieved by parenteral, particularly

intravenous administration, this procedure should probably be reserved for specific indications, since it introduces some of the hazards common to all injections made directly into the blood stream. One of these is anaphylactic shock. Rare cases of such incidents have been reported following the intravenous injection of thiamin. In our laboratory we have investigated the sensitizing effect of thiamin in dogs and guinea pigs. Ten dogs were divided into two groups. One group received on 10 consecutive days 50 mgm. per kgm. of thiamin hydrochloride intravenously, an amount sufficient to produce severe toxic reactions such as convulsive stimulation, nausea, vomiting and collapse. After 10 daily injections, an interval of 10 to 20 days was allowed before the final experiment was performed. The second group of dogs received no pretreatment. There was no significant difference between the two groups. However, upon autopsy it appeared that in some of the dogs which had received previously 10 doses of thiamin, the right heart was dilated and

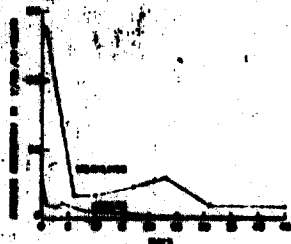


Fig. 3. Urinary excretion of pantothenic acid in weanling and adult dogs during depletion.

the abdominal organs showed greater congestion, while this was not observed in any of the controls. The outcome of the guinea pig experiments was not conclusive.

Normal as well as depleted animals excrete a certain amount of the administered vitamin through the urine. Both the absolute amount and the percentage of excretion (with or without administration of a test dose) have been used as a measure of the state of relative saturation and as indicator of the degree of deficiency. It is in the urine, where the first sign of vitamin depletion can usually be found (fig. 3). However, it must be remembered, that without the use of a test dose the urinary level may merely be a measure of the immediately preceding vitamin intake. In order to be significant, such "saturation tests" should therefore be conducted in fasting animals and after administration of a test dose.

In contrast to the wide fluctuations in the vitamin content of the urine, the concentration in the blood remains usually remarkably constant and such determinations offer therefore little help in the diagnosis of early vitamin deficiencies.

While the difference between the amount of a vitamin administered and excreted is not necessarily an indicator of the maintenance dose, it can nevertheless serve as a guide for the determination of the appropriate therapeutic dose. This is shown in the following experiment, in which the same dose of pantothenic acid was daily given to pantothenic acid depleted dogs and its urinary excretion determined. It may be seen that at least 4 to 10 times the maintenance dose (previously determined in actual feeding experiments) had to be given over a period of 1 to 2 weeks until saturation was accomplished. This again emphasizes the justification for apparently excessive vitamin doses in cases of advanced deficiencies.

Other factors which may considerably affect the dosage of vitamins are diet and age. For example, thiamin is necessary for the utilization of carbohydrates and consequently larger doses are needed when the carbohydrate intake is high.

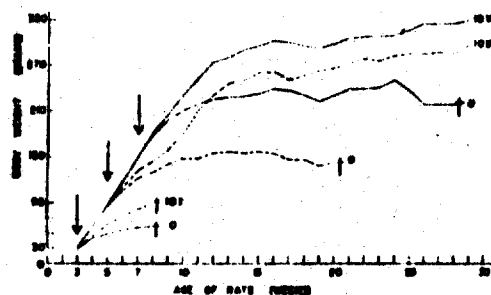


Fig. 4. Influence of age on pantothenic acid requirement. ↓ indicates when groups were changed from full diet to depletion diet. ↑ indicates death of 50 per cent of the animals in group.

Similarly, it has been reported that the rat requirement for riboflavin or pyridoxine increases when a high fat diet is given.

The influence of age upon vitamin requirements depends upon the specific vitamin studied. Generally, a growing organism requires relatively larger amounts than an adult. This is particularly true for pantothenic acid where with increasing age a striking decrease takes place not only in the relative, but also in the absolute requirement (15) (fig. 4).

In the preceding paragraphs the general toxicity of vitamins and their absorption, storage and excretion have been discussed, but nothing has as yet been said about their pharmacodynamic properties. The reason for this is that in healthy animals vitamins in doses below the toxic range fail to exhibit such effects. Nevertheless, in a vitamin depleted animal they are capable of restoring to normal the pathological changes caused by lack of a specific vitamin and under

such conditions they compare in potency favorably with the most powerful medicinal agents, having a marked effect in doses within the milligram range.

The high specificity of vitamins can easily be demonstrated by comparing their effect upon pathological changes which, although closely resembling each other, are due in one instance to a specific vitamin deficiency and in the other to a non-specific cause. This is illustrated in the following experiment (Fig. 3): Of two groups of rats one was placed on a thiamin deficient diet, while the other receiving a complete diet was restricted in its food intake to that of the depletion group. In both groups frequent records were taken of the weight, the heart rate and the sensitivity to a convulsing dose of thujone. It may be seen that in both groups convulsions could be produced more readily as the experiment progressed. However, only in the thiamin deficient group did the heart rate show the typical retarda-

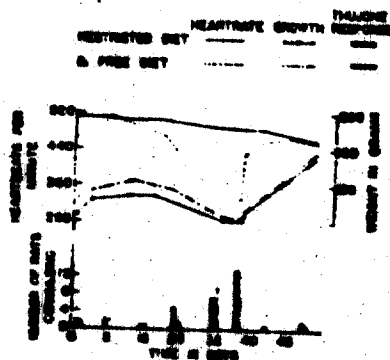


Fig. 3. The influence of thiamin on heart rate, growth and response to thujone.

tion, which responded immediately to thiamin administration. The increased sensitivity to the convulsant drug was, on the other hand, a result of the general state of inanition and responded therefore in both groups with equal promptness to an increased intake of food (16).

The observation of A. Keys (17) that administration of vitamins is without influence on the performance of athletes already living on an adequate diet, can be cited as further evidence that vitamins exhibit their effect only in specific deficiencies. However, if the subjects in Keys' experiment would have lived on a suboptimal vitamin intake, a beneficial effect of vitamins on the endurance might have been found. In our laboratory such an effect could be demonstrated in vitamin B₁ deficient rats. In experiments still in progress the endurance of rats in various stages of vitamin deficiencies is being measured by their ability to carry a given load while swim-

ming. During thiamin deficiency, the work performance decreases to a very low level; administration of the vitamin improves it greatly, even if the food intake remains restricted (table 6).

A decrease in endurance due to a suboptimal intake of vitamins is but one of the many appar-

TABLE 6
Effect of thiamin-depletion on endurance

RAT NO.	WEIGHT	DATE	SWIMMING TIME	REMARKS	REMARKS
	grams		minutes		
112	30	1/25	0	10% Thiamin 4 gm. food	
	35	1/26	12	4% Thiamin 5 gm. food	Fed and dead after swim- ming
	38	1/26	14	4% Thiamin 5 gm. food	
114	30	1/25	0	10% Thiamin 4 gm. food	
	35	1/26	16	4% Thiamin 5 gm. food	Fed and dead after swim- ming
	38	1/26	20+	4% Thiamin 5 gm. food	
119	30	1/25	0	10% Thiamin 4 gm. food	
	35	1/26	20+	4% Thiamin 4 gm. food	Fed and dead after swim- ming
	38	1/26	20+	4% Thiamin 4 gm. food	
SWE-1	30	2/25	5	10% Thiamin food ad lib.	
	30	2/26	4	4% Thiamin food ad lib.	Control
	30	2/26	2	4% Thiamin food ad lib.	
SWE-2	30	2/21	25	10% Thiamin food ad lib.	
	30	2/26	10	4% Thiamin food ad lib.	Control
	30	2/26	20	4% Thiamin food ad lib.	

ently non-specific conditions in which administration of vitamins may prove to be an effective therapeutic measure. While the clinical symptomatology of fully developed vitamin deficiencies, such as rickets, scurvy, beriberi and pellagra, is well known, there are undoubtedly a far greater number of subclinical deficiencies and of other

pathological conditions, in which vitamin deficiency is a complicating factor. The latter respond to vitamin therapy to the extent to which the deficiency is eliminated. Thus, it has frequently been stated that thiamin administration

TABLE 7

Functional and metabolic activity of liver of mice in heat and influenza

REFLECTED BOAGE				REFLECTED BOAGE				REFLECTED BOAGE				REFLECTED BOAGE				REFLECTED BOAGE					
No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Average
mgm	17.5	17.5	14.5	13.5	20.5	9.5	14.5	16.5	19.5	15.5	17.5	11.5	10.5	15.5	12.5	17.5	17.1	15.5	11.5		
% per gram	80	71	50	50	50	51	52	50	50	50	50	51	45	50	50	50	52	54	50	50	
% per gram	21	20	20	20	20	20	20	20	20	20	20	20	20	20	20	20	27	31	30	30	
Average																					

REFLECTED BOAGE				REFLECTED BOAGE				REFLECTED BOAGE				REFLECTED BOAGE				REFLECTED BOAGE					
No.	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	Average
mgm	15.5	17.5	13.5	12.5	12.7	9.1	10.2	10.3	10.5	12.1	10.5	10.2	11.5	12.5	13.0	12.2	15.9	10.5			
% per gram	70	80	70	66	64	50	70	63	59	57	65	70	66	64	54	66	77	60			
% per gram	25	29	30	28	23	23	29	24	25	29	27	26	28	26	22	21	25	34			
Average																					

in diabetes mellitus permits a reduction in the necessary dosage of insulin. However, in strictly controlled experiments it can be shown that thiamin itself does not influence the course of the disease; the beneficial effect is probably due to the

correction of a mild deficiency which is often produced by imposition of a rigid diet. Similarly, administration of vitamins to patients with gastric ulcers or on other restricted diets may be indicated and may improve the general condition by correcting a nutritional deficiency.

Under certain conditions, as in lactation, pregnancy, tropical environments (increased thiamin excretion through sweating) and infectious diseases, the vitamin requirements are considerably greater than normal. Thus, it has been reported by Mills (18) that the thiamin requirements of rats kept in a tropical climate are about twice those of the control animals. This is due primarily to a decreased food consumption in the hot room and can be corrected by doubling the thiamin concentrations in the diet. Mice on a sub-optimal riboflavin intake are less resistant to pneumococcus I infection (19); and in our own laboratory it has been found that during an infection with influenza virus the pantothenic acid content of the liver is significantly reduced, while the riboflavin content remains constant (table 7).

In summary, it may be stated that vitamins are pharmacological agents of extremely low toxicity which exhibit a pharmacodynamic effect only in an animal depleted of the specific vitamin. However, since mild stages of vitamin deficiencies are quite frequent and may accompany pathological conditions resulting from other causes, vitamins have a wide field of therapeutic application.

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Effects of Increased Doses of Natural and Synthetic Vitamin B₁.

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The extremely large span between the therapeutic and the toxic dose of vitamins has resulted in the fact that the pharmacology and the pathology of the avitaminoses were investigated much earlier than those of the hypervitaminoses. Thus, the disease conditions caused by the lack of vitamin B₁ have been known for a long time whereas there is no detailed information as yet on the toxic effects through overdosing. Although this vitamin was chemically isolated by Jansen and Donath¹⁾ as early as 1927, it was only accessible in such small quantities that pharmacological and especially toxicological series tests with their extreme requirements for material could not be begun. Only after R.R. Wilson et al²⁾ had developed a process which rendered possible the production of a pure vitamin from rice bran with a better yield, the pharmacological and clinical examination of the crystallized vitamin B₁ was started. But even then, the tests - because of the high cost of the material (in the fall of 1935, the production costs for 1 gram were more than \$ 400.-) were limited to the substitute of vitamin B₁ extracts by the pure vitamin and the examination of its effect in relieving experimental and clinical vitamin B₁ deficiency symptoms. However, these limited works showed already that the therapeutical range of the pure vitamin B₁ was not smaller than that of the other vitamins used in their pure form. Thus, Williams, Waterman and Vorhaus³⁾ report on subcutaneous injections of individual doses of up to 90 mg in the human being without the least secondary effects. Last year, Moll⁴⁾ reported here that white mice tolerate well intravenous, intraperitoneal

and subcutaneous injections of 0,5 ccm of a 1:1000 vitamin solution, and that the intravenous injection of 0,5 ccm of a 0,5% solution causes an acute shock effect but that the animals recover from it in a few minutes.

An examination of the acute toxic effects of pure vitamin B₁ in series tests only became possible after the question of its manufacture had been solved and especially after Williams and Cline ⁵⁾ had succeeded in synthetically producing the valuable substance in a relatively simple process.

This publication is a short report of our investigations on the acute toxic effects of pure vitamin B₁.

Toxic Effects on Rats and Mice

The tolerance of crystallized vitamin B₁ was first tested on rats and mice. The weight of the animals which all came from the same breeder and received a uniform normal diet, was, in the case of the mice 18 - 20 grams, in the case of the rats, 95- 105 grams; males were used almost exclusively. The animals were kept in an air-conditioned space, where the experiments were carried out; the temperature was 23°C ($\pm 1^\circ$), the relative humidity 45% ($\pm 3\%$). All experiments were made during the months of September, October and November. The injections were made either under the skin of the abdomen, or, in the case of the rats into the left vena saphena, in the case of the mice into the tail vein. In the case of intravenous injections, the speed was never more than 0,5 ccm per minute. The concentration of the solutions alternated between 1,5 and 10%, according to the total quantity to be introduced.

Table 1 shows the results of these tests in a clear arrangement. Doses of up to 100 mg/kg subcutaneously and 10 mg/kg intravenously are tolerated by rats without reaction. The injection of larger quantities is followed by severe disease symptoms which begin with a severe loss of vital power. If the injection is interrupted at this point, the animals usually recover within 10-15 minutes and then tolerate a new intravenous injection, as already described by Moll. Thus, it was possible to inject intravenously up to 6,5 mg of the vitamin in individual

doses of 1 mg in 1% solution over a period of one and a half hours without causing death, whereas in the case of a continuous injection, 2,5 mg of the same solution are fatal. The loss of vital power is followed by general muscle tremor and occasional convulsive movements if the intravenous injection is continued, or lethal or sublethal quantities are injected subcutaneously. Breathing becomes labored and irregular and death is apparently caused by respiratory arrest while the heart continues to beat regularly for some time. Death occurs not later than 15 - 20 minutes after the injection; if the animal survives this period of time, it always completely recovers within the next 24 hours.

Since there was the possibility that the relatively strong acid reaction (p_H 3,5) of the solutions might be the cause for the rapid collapse, control tests were made with injections of a vitamin solution buffered to p_H 6,6 on one hand, and the buffer solution alone, on the other hand. As shown in Table 1, these control tests have no influence on the final result. The definitely lethal vitamin B_1 doses, in the case of intravenous administration are 2,5 mg per mouse and 30 mg per rat, in the case of subcutaneous injection 15 mg per mouse and 180 mg per rat. While there is a definite difference in the toxic sensitivity between mice and rats, the ratio between the intravenously and subcutaneously lethal quantity remains strikingly the same, namely 1:6.

The peroral toxicity was determined in mice who were fed vitamin B_1 in 10 or 20% solutions by means of a throat probe. Administrations of up to 75 mg per 20 g mouse in a 10% solution remained without effect. However, 100 mg in the form of the 20% solution were lethal within 10 - 15 minutes with the same symptoms which were observed after intravenous or subcutaneous poisonings. However, since the possibility could not be rejected that the death could be connected with the relative large fed quantity of the clearly acid or hypertonic solution, we gave to test mice on one hand up to 1 ccm of a buffer solution of p_H 3,5, on the other hand, up to 0,5 ccm of a saturated cooking salt solution. The feeding of the acid solution alone had no

consequences. Also, the mice which had received the saturated cooking salt solution remained apparently healthy during the next 4 -5 hours and at least showed no symptoms which would be at all similar to a vitamin B₁ poisoning; but they were found dead the following morning.

In no experiment was there any difference in toxicity between the natural and the synthetic vitamin B₁; this is in complete agreement with their identical behavior in the therapeutic application.

Toxic Effects on Rabbits, Dogs and Monkeys

In another test series, we examined the toxicity of intravenously injected vitamin B₁ on rabbits, dogs and monkeys. The tests were carried out partly on unaenesthetized and partly on urethanized animals - the latter if they were connected with a recording of respiration, blood pressure and leg volume. In all experiments, a 10% vitamin solution was injected intravenously. The injection speed alternated in the individual tests between 0,1 ccm and 0,5 ccm per minute, but was maintained the same during the whole duration of the test. The lethal quantity was the same for aenesthetized and unaenesthetized animals, but was very dependent on the injection speed: In the case of faster injections, death occurred already at smaller vitamin doses. A similar behavior had already been observed in mice and rats where an increase of the injection speed from 0,1 ccm to 0,2 ccm per minute resulted in a 25% increase of the toxicity.

One of the first signs of the vitamin B₁ poisoning in the acute experiment is a change in respiration which slows down and becomes irregular and frequently assumes the Cheyne-Stokes character (Illustration 1). Until shortly before the end, the blood pressure remains unchanged, and the heart beat is strong and regular, even at a time when there are only sporadic respiratory movements with the aid of the auxiliary muscles. Usually it is only after the complete arrest of the respiration that the blood pressure drops abruptly (Ill. 2).

However, at this time, the heart still beats regularly; its arrest only takes place 1-2 minutes after the respiratory arrest.

The intravenous doses necessary to cause death were 210 mg in rabbits, weighing 700 and 730 g; and 1600 mg in dogs weighing 3 and 3,7 kg. Intravenous injections of 100 ccm of a 0,1 n hydrochloric acid solution resulted in no obvious changes of respiration and blood pressure in a control dog.

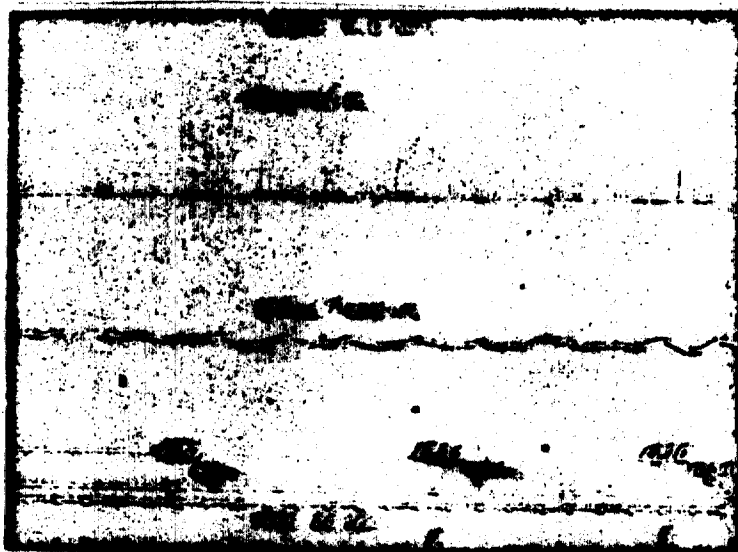


Illustration 1

Dog, 3 kg, urethan anaesthesia (1,3 g/kg)

Upper curve: Respiration

Lower curve: Carotid blood pressure

Time recording: Every 20 seconds

Continuous infusion of a 10% vitamin B₁ solution into the left jugular vein at a speed of 0,15 ccm per minute.

To mark 1: 1170 mg infused

To mark 2: 1230 mg infused

To mark 3: 1270 mg infused

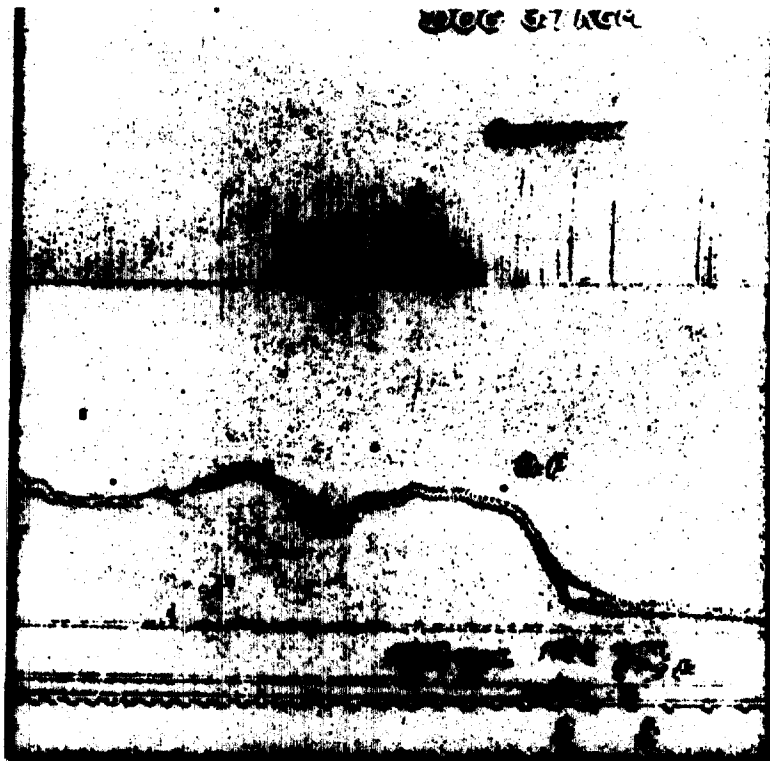


Illustration 2

Dog, 3,7 kg, urethan anaesthesia (1,5 g/kg)

Upper curve: Respiration

Lower curve: Carotid blood pressure

Time recording: Every 20 seconds

Continuous infusion of a 10% vitamin B₁ solution into the left jugular vein at a speed of 0,6 ccm/minute

To mark 1: 1000 mg infused

To mark 2: 1100 mg infused

To mark 3: Infusion terminated.

Occasionally, a peripheral vascular dilatation was observed after intravenous injection of very large but not lethal doses of vitamin B₁ which could be followed very clearly in the ear vessels of the unanaesthetized rabbit by means of the photo- and thermoelectrical method mentioned by Molitor and Kniazuk⁶⁾ Illustration 3 shows a section of such an experiment.

Repeated injection of a buffered solution of p_H 3,5 did not show such a change (Illustration 4).



Illustration 3

Rabbit, 3 kg

Photocell and thermo-element at the left ear

Upper curve: (V.R.) Changes of the amount of light penetrating through the ear, according to the changes in blood flow.

Lower curve: (Temp.) Changes of the temperature of the skin

Time recordings: Every 15 seconds

Between mark 1 and mark 2, intravenous injection of 200 mg Vitamin B₁.

Mark 3: Measuring of the temperature of the skin (33,8°C).

Mark 4: Measuring of the temperature of the skin (36,2°C).

Mark 5: Measuring of the temperature of the skin (35,9°C).



Rabbit, 3 kg

Photocell and thermo-element at the left ear

Upper curve: (V.R.) Changes of the amount of light penetrating through the ear according to the changes in the blood flow.

Lower curve: Changes of the temperature of the skin

Time recordings: Every 15 seconds

Between mark 1 and mark 2, intravenous injection of 2 ccm of a buffered solution of p_H = 3,5.

In some experiments, we had the impression that injections of large doses of vitamin B₁ led to an increased nervous irritability of the animals. Thus rats, which were in a metabolism apparatus according to Richards and Collison, after subcutaneous injection of large quantities of vitamin B₁ usually showed a significantly more agitated curve than in the immediately preceding period of time (Illustration 5). The basal metabolism in this case was not significantly changed. A similar increase in irritability was also frequently found in rabbits in which the action of the ear vessel reflexes shows the degree of the nervous irritability. As shown in illustration 6, the curves of the blood flow in the skin and the skin temperature in the normal rabbit are almost straight lines and only show a sudden change if the animal is subjected to strong stimulation of the senses. However, after the intravenous injection of 200 mg of vitamin B₁, the picture changes. As a result of the generally increased reaction of the nervous system both curves, unrelated to specific stimulations of the senses, fluctuate up and down almost continuously.

Even though the just described changes of the vascular and nervous system do not occur with such regularity that we can prove them to be typical concomitant symptoms of the overdosis of vitamin B₁, we still think they should be mentioned, especially since control experiments with solutions of the same hydrogen-ion concentration were negative.

Local Irritations Caused by Vitamin B₁

Several types of animals were tested in regard to local irritations in the usual manner. The methods used were intracutaneous^{injections} into the abdominal skin of guinea pigs and into the superficial tissue layers of rabbit ears, subcutaneous injections under the skin of the backs of monkeys and rats, intramuscular injections into the gluteal muscular system and the muscular system of the thighs of dogs and instilling into the eyes of rabbits and cats. The vitamin solutions were of 1, 5 and 10% and were intentionally unbuffered but left in their natural hydrogen-ion concentration of p_H 3.5.



Illustration 5

Rat, 125 g

Upper curve: Basal metabolism before vitamin B₁ injection

Lower curve: Basal metabolism after subcutaneous injection of 80 mg vitamin B₁

Time recordings: Every 5 seconds

Each tooth corresponds to an oxygen consumption of 1,8 ccm

Control tests with a buffered solution of p_H 3,5 were made in all cases, containing 0,10 n sodium chloride, 0,10 n aminoacetic acid and 0,10 n hydrochloric acid. The used vitamin B₁ solutions were sterilized for 30 min. at 120°C in the autoclave. The biological evaluation of the thus treated solutions had shown that there was no difference in their effectiveness. The minor irritations which immediately followed the intracutaneous injection of 0,1 ccm of the respective solutions were the same in the case of vitamin or control solutions. However, while the irritations caused by the latter had completely disappeared after 24 hours, they were still present at that time in the case of the vitamin solutions and healed within a few days forming scabs.

Subcutaneous injections of 0,5 ccm of the 1%, 5% and 10% vitamin solutions in rats caused a pronounced local irritation which on the next day led to the formation of blisters and to extensive losses of epitheliums. Similar effects, although they were not as pronounced were observed in rabbits, while in monkeys and dogs they were hardly visible and were limited to a slight redness which lasted 12-24 hours.



Illustration 6a

Rabbit, 2,8 kg

Photocell and thermoelement at the left ear

Upper curve: (V.R.) Changes of the quantity of light penetrating the ear, corresponding to changes in the blood flow.

Lower curve: (Temp.) Changes in the temperature of the skin

Time recordings: Every 15 seconds

1- 7 stimulations of smell

Intramuscular injections were tolerated by monkeys as well as by dogs without the slightest subjective or objective symptoms of irritation.

In none of the used types of animals was there an occurrence of thrombosis after intravenous injection.

Instilling 10% vitamin B₁ solutions into the eyes of cats and rabbits resulted in minor irritations which, however, were not more pronounced than after instilling a control solution containing no vitamin of the same hydrogen-ion concentration.

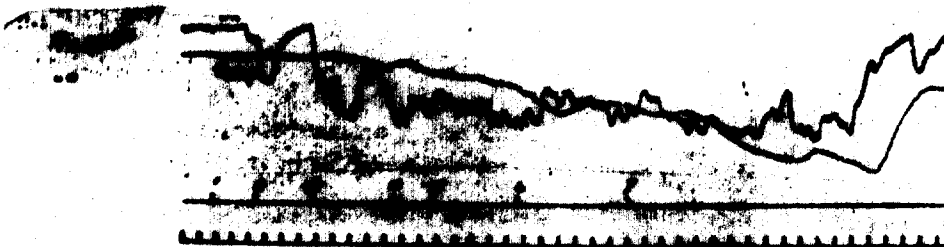


Illustration 6b

(After intravenous injection of 200 mg Vitamin B₁)

1. Measurement of skin temperature (37,6°C).
2. and 3. Opening the door of the testing room and walking around
4. Stimulations of smell
5. Measuring the skin temperature (36,3°C)
6. Stimulation of smell

Discussion

Our experiments showed that pure vitamin B₁ in extremely large quantities can result in acutely pronounced pathological changes and death. However, the required doses in such a case are so exaggerated that one is justified in pronouncing Vitamin B₁ as completely non-toxic for the practical application. The following consideration, the mathematical part of which is based on the formulas on the vitamin B₁ requirement supplied by Cowgill ⁷⁾, will show the extremely large gap between a therapeutically effective and a toxic dose. The daily required minimum quantities of vitamin B₁ are for mice 4 micrograms, for rats 6 micrograms, for dogs 18 micrograms. In contrast, the intravenously lethal doses are for mice 2500 micrograms, for rats 30 000 micrograms and for dogs between 1 200 000 and 1 600 000 micrograms. Thus, the intravenously lethal dose is 600 times larger in the case of mice, 5000 times larger in the case of rats and 67 000 times larger in the case of dogs than the therapeutic one. If one also takes into account that the subcutaneously lethal dose in the case of mice and rats is six times higher, and the perorally lethal dose in the case of mice is forty times higher than the intravenously lethal dose, the above mentioned opinion in regard to the practically acute non-toxicity of vitamin B₁ is not unjustified. However, this opinion is only based on animal experiments which, for the most part, were carried out on small animals. However, in this connection, the fact must be stressed that the toxicity of vitamin B₁ per unit and body weight is the largest especially in small animals, while it rapidly decreases with the increasing size of the animal. Intravenous injections of 1 g vitamin B₁ each in a 10% solution in two rhesus monkeys weighing 5,5 kg, at an injection speed of 2 ccm per minute were accompanied by no pathological symptoms.

The above described experiments and results at present only refer to the acute toxicity test and must, for judging a remedy which is mainly intended to be taken over a longer period of time, be supplemented by the determination of the

toxicity in the case of a chronic administration. Such experiments have now been carried out for over a year and already show that clinically detectable damages do not occur and that the vitamin B₁ has a very large therapeutical range. In the near future, these tests will be discussed here in greater detail.

Conclusions

1. The acute lethal doses of natural or synthetic vitamin B₁ on intravenous injection in mice are 125 mg/kg, in rats 250 mg/kg, in rabbits 300 mg/kg, in dogs 350 mg/kg. By subcutaneous administration the lethal dose is 6-fold, by peroral administration 40-fold that of the intravenous injection.
2. When lethal quantities of vitamin B₁ are administered, the respiratory arrest precedes the cardiac arrest. The heart beats strongly and regularly to the end. The outward signs of the poisoning are shock, muscle tremors, occasional clonic cramps and respiratory disturbances.

Toxicity of natural and synthetic vitamin B₁ in rats in mice

Type of animal	Material	1 Dose in mg.	Intravenous 2 Total No. of animals	3 Of these sur- viving	subcutaneous			Peroral		
					1	2	3	1	2	3
Mouse	Synthetic	2.3	2	2	10	4	4			
"	"	2.5	3	1	12	2	2			
"	"	2.75	2	0	15	2	0			
"	Natural	2.0	6	6	10	5	5	75	3	3
"	"	2.5	8	2	12	5	4	100	2	0
"	"	3.0	3	0	15	4	0	130	2	0
"	Buffered pH 6.6	2.3	2	2	10	3	3			
"	"	2.5	2	0	12	4	4			
"	"	3.0	2	0	15	2	0			
Rat	Natural	10	3	3	100	6	6			
"	"	20	6	5	130	2	2			
"	"	25	3	1	150	2	2			
"	"	30	2	0	180	2	1			
"	"				200	3	1			
"	Synthetic	10	2	2						
"	"	20	3	3						
"	"	25	3	1						

3. Vitamin B₁ solutions can be sterilized in the autoclave at

120°C for 30 minutes without losing their effectiveness.

4. Repeated intravenous and intramuscular injections of vitamin B₁ were tolerated by all animals (mice, rats, guinea pigs, rabbits, dogs, monkeys) without the slightest subjective or objective irritations. Intracutaneous and subcutaneous injections in rats and guinea pigs caused pronounced local irritations whereas they were absent in rabbits, dogs and monkeys.

5. Pure vitamin B₁ has such an extremely large therapeutical range that for all applicable therapeutical purposes it can be considered to be practically non-toxic.

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Über die Wirkungen hoher Gaben von natürlichem und synthetischem Vitamin B₁.

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Die außerordentlich große Spanne zwischen der Heil- und Gift-dosis von Vitaminen hat es mit sich gebracht, daß die Pharmakologie und Pathologie der Avitaminosen viel früher erforscht wurde als die der Hypervitaminosen. So sind denn auch die durch Fehlen von Vitamin B₁ hervorgerufenen Krankheitszustände seit langem wohlbekannt, während über Giftwirkungen durch Überdosierung noch keine eingehenden Mitteilungen vorliegen. Obwohl dieses Vitamin bereits 1927 von Jansen und Donath¹⁾ chemisch isoliert wurde, war es doch nur in so geringen Mengen zugänglich, daß an pharmakologische und insbesondere toxikologische Reihenversuche mit ihrem außerordentlichen Materialaufwand nicht geschritten werden konnte. Erst seitdem R. R. Williams und Mitarbeiter²⁾ ein Verfahren entwickelt hatten, welches die Reindarstellung aus Reiskleie mit einer besseren Ausbeute ermöglichte, wurde mit der pharmakologischen und klinischen Prüfung des kristallisierten Vitamins B₁ begonnen. Aber auch dann beschränkten sich infolge der Kostbarkeit des Materials (beliefen sich doch noch im Herbst 1935 die Herstellungskosten von 1 Gramm auf über 400 Dollar) die Versuche auf den Ersatz von Vitamin B₁-Extrakten durch das reine Vitamin und die Prüfung seiner Wirksamkeit in der Behebung von experimentellen und klinischen Vitamin B₁-Mangelercheinungen. Aus den spärlichen Arbeiten ging aber bereits hervor, daß die therapeutische Breite des reinen Vitamins B₁ nicht geringer war als die der anderen reindargestellten Vitamine. So berichten Williams, Waterman und Vorhaus³⁾ über subkutane Injektionen von Einzeldosen bis zu 90 mg beim Menschen ohne die geringsten

Nebenercheinungen. Moll¹⁾ hat im Vorjahre an dieser Stelle mitgeteilt, daß weiße Mäuse intravenöse, intraperitoneale und subkutane Injektionen von 0,5 ccm einer Vitaminlösung 1:1000 gut vertragen, und daß die intravenöse Injektion von 0,5 ccm einer 0,5%igen Lösung zwar eine akute Schockwirkung nach sich zieht, die Tiere sich von ihr aber in wenigen Minuten erholen.

Eine Untersuchung der akuten Giftwirkungen von reinem Vitamin B₁ in Reihenversuchen wurde erst möglich, seitdem die Frage seiner fabrikmäßigen Herstellung gelöst worden war und insbesondere, seitdem es Williams und Cline²⁾ gelungen war, den kostbaren Stoff auf relativ einfache Weise synthetisch darzustellen.

Die vorliegende Veröffentlichung ist ein kurzer Bericht unserer Untersuchungen über die akuten Giftwirkungen von reinem Vitamin B₁.

Giftwirkungen an Ratten und Mäusen.

Die Verträglichkeit von kristallisiertem Vitamin B₁ wurde zunächst an Ratten und Mäusen geprüft. Das Gewicht der Tiere, die alle aus der gleichen Zucht stammten und eine gleichförmige Normalkost erhielten, betrug bei Mäusen 18—20 Gramm, bei Ratten 95—105 Gramm; es kamen fast ausschließlich Männchen zur Verwendung. Die Tiere wurden in einem künstlich klimatisierten Raum gehalten und daselbst auch die Versuche vorgenommen; die Temperatur betrug 23° C ($\pm 1^\circ$), die relative Feuchtigkeit 45% ($\pm 3\%$). Alle Versuche wurden während der Monate September, Oktober und November angestellt. Die Einspritzungen wurden entweder unter die Bauchhaut oder, bei Ratten, in die linke Vena saphena, bei Mäusen in die Schwanzvene vorgenommen. Bei intravenösen Injektionen betrug die Geschwindigkeit nie mehr als 0,5 ccm je Minute. Die Konzentration der Lösungen wechselte zwischen 1, 5 und 10%, je nach der Gesamtmenge des einzuführenden Materials.

Tabelle 1 gibt die Ergebnisse dieser Versuche in übersichtlicher Form wieder. Dosen bis zu 100 mg/kg subkutan und 10 mg/kg intravenös werden von Ratten reaktionslos vertragen. Einspritzung noch größerer Mengen ist von schweren Krankheitserscheinungen gefolgt, die mit einem schweren Schwächeanfall beginnen. Wird zu diesem Zeitpunkt die Injektion abgebrochen, so erholen sich

gewöhnlich die Tiere innerhalb 10—15 Minuten und vertragen dann eine neuerliche intravenöse Injektion, wie dies bereits Moll beschrieben hat. So gelang es, bis zu 6,5 mg Vitamin in Einzeldosen von 1 mg in 1%iger Lösung im Verlauf von anderthalb Stunden intravenös zu injizieren, ohne den Tod herbeizuführen, während bei ununterbrochener Injektion 2,5 mg der gleichen Lösung tödlich sind. Allgemeines Muskelzittern und gelegentliche krampfartige Zuckungen folgen dem Schwächeanfall, wenn die intravenöse Injektion fortgesetzt wird, oder letale oder subletale Mengen subkutan injiziert werden. Die Atmung wird mühsam und unregelmäßig, und der Tod erfolgt anscheinend durch Atemstillstand, während das Herz noch regelmäßig für einige Zeit weiterschlägt. Der tödliche Ausgang tritt nicht später als 15—20 Minuten nach der Injektion ein; überlebt das Tier diese Zeitspanne, so erholt es sich regelmäßig vollkommen innerhalb der nächsten 24 Stunden.

Da die Möglichkeit bestand, daß die verhältnismäßig stark saure Reaktion (p_H 3,5) der Lösungen die Ursache des raschen Kollapses sei, wurden Kontrollversuche mit Injektionen einerseits einer auf p_H 6,6 gepufferten Vitamin-Lösung, andererseits der Pufferlösung allein angestellt. Wie aus Tabelle 1 ersichtlich ist, sind diese Kontrollversuche ohne Einfluß auf das Endergebnis. Die sicher tödlichen Vitamin B_1 -Dosen sind bei intravenöser Beibringung 2,5 mg pro Maus und 30 mg pro Ratte, bei subkutaner Injektion 15 mg pro Maus und 180 mg pro Ratte. Während ein deutlicher Unterschied in der Vitamin B_1 -Giftempfindlichkeit zwischen Mäusen und Ratten besteht, bleibt das Verhältnis zwischen der intravenösen und subkutan tödlichen Menge für beide Tierarten auffallend gleich, nämlich 1:6.

Die perorale Toxizität wurde an Mäusen bestimmt, denen Vitamin B_1 in 10 oder 20%igen Lösungen mittels Schlundsonde verfüttert wurde. Gaben bis zu 75 mg je 20 g Maus in 10%iger Lösung blieben ohne Wirkung. Dagegen führten 100 mg, in Form der 20%igen Lösung, innerhalb von 10—15 Minuten den Tod unter den gleichen Erscheinungen herbei, die nach intravenösen oder subkutanen Vergiftungen beobachtet worden waren. Da aber die Möglichkeit nicht von der Hand gewiesen werden konnte, daß der tödliche Ausgang im Zusammenhang mit der relativ großen verfütterten Menge der deutlich sauren, bzw. hypertonischen Lö-

sung stünde, verabfolgten wir Kontrollmäusen einerseits bis zu 1 ccm einer Pufferlösung von p_H 3,5, andererseits bis zu 0,5 ccm einer gesättigten Kochsalzlösung. Verfütterung der Säurelösung allein zog keinerlei Folgen nach sich. Auch die Mäuse, welche die gesättigte Kochsalzlösung erhalten hatten, blieben anscheinend während der nächsten 4—5 Stunden gesund und zeigten jedenfalls keinerlei Symptome, die auch nur im Entferntesten an das Bild einer Vitamin B_1 -Vergiftung erinnert hätten; sie wurden aber am folgenden Morgen tot aufgefunden.

In keinem Versuch ergab sich ein Unterschied in der Giftigkeit zwischen natürlichem und künstlichem Vitamin B_1 ; dies steht in völliger Übereinstimmung mit ihrem identischen Verhalten im Heilversuch.

Giftwirkungen an Kaninchen, Hunden und Affen.

In einer weiteren Versuchsreihe prüften wir an Kaninchen, Hunden und Affen die Giftigkeit von intravenös injiziertem Vitamin B_1 . Diese Versuche wurden teilweise am unbetäubten, teilweise am urethranisierten Tier angestellt, — letzteres dann, wenn es mit einer Registrierung von Atmung, Blutdruck und Beinvolumen verbunden waren. In allen Versuchen wurde eine 10%ige Vitaminlösung intravenös injiziert. Die Injektionsgeschwindigkeit wechselte bei den einzelnen Versuchen zwischen 0,1 ccm und 0,5 ccm pro Minute, wurde aber während der ganzen Versuchsdauer gleich gehalten. Die tödliche Menge war für betäubte und unbetäubte Tiere gleich, zeigte aber große Abhängigkeit von der Injektionsgeschwindigkeit, indem bei rascherer Einspritzung der Tod schon bei kleineren Vitamin-Dosen erfolgte. Ein ähnliches Verhalten hatte sich übrigens auch an Mäusen und Ratten beobachten lassen, wo eine Erhöhung der Injektionsgeschwindigkeit von 0,1 ccm auf 0,2 ccm pro Minute zu einer 25%igen Steigerung der Giftigkeit führte.

Eines der ersten Anzeichen der Vitamin B_1 -Vergiftung ist im akuten Versuch eine Änderung der Atmung, die verlangsamt und unregelmäßig wird und häufig Cheyne-Stokes'schen Typus annimmt (Abb. 1). Der Blutdruck bleibt bis knapp vor dem Ende unverändert, und das Herz schlägt kräftig und regelmäßig, selbst noch zu einem Zeitpunkte, in dem nur mehr vereinzelte

Atembewegungen mit Zuhilfenahme der Auxiliarmuskulatur erfolgen. Gewöhnlich kommt es erst nach dem völligen Aussetzen der Atmung zu einem jähen Absinken des Blutdrucks (Abb. 2). Auch in diesem Zeitpunkte schlägt aber das Herz noch regelmäßig; sein Stillstand tritt erst 1—2 Minuten nach dem Atemstillstand ein. Die zur Herbeiführung des Todes erforderlichen

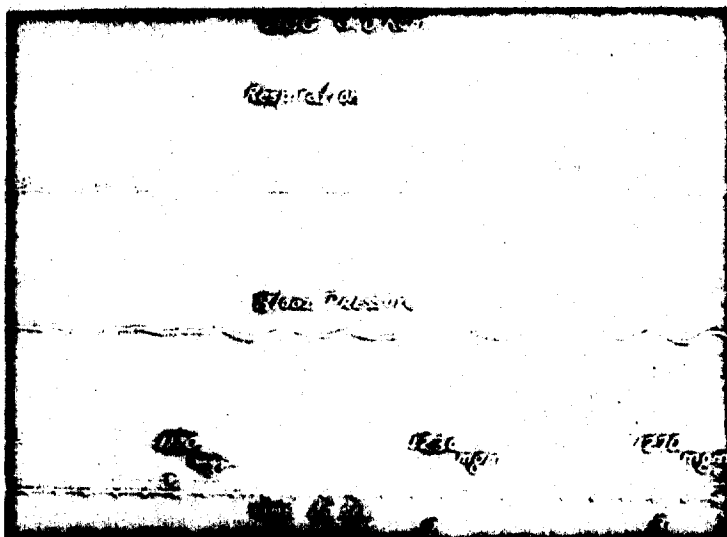


Abb. 1.

Hund, 3 kg. Urethan-Narkose (1,3 g/kg).

Obere Kurve: Atmung.

Untere Kurve: Karotis-Blutdruck.

Zeitmarkierung alle 20 Sekunden.

Kontinuierliche Infusion einer 10%igen Lösung von Vitamin B₁ in die linke Jugularvene mit einer Geschwindigkeit von 0,15 ccm/Minute.

Die Marke 1: 1170 mg infundiert.

Die Marke 2: 1230 mg infundiert.

Die Marke 3: 1270 mg infundiert.

intravenösen Dosen waren 210 mg bei Kaninchen von 700 g, bzw. 730 g; und 1200 mg, bzw. 1600 mg bei Hunden von 3 kg, bzw. 3,7 kg. Intravenöse Injektionen von 100 ccm einer 0,1 n Salzsäurelösung zogen bei einem Kontrollhund keine auffälligen Veränderungen von Atmung und Blutdruck nach sich.

Gelegentlich wurde eine periphere Gefäßerweiterung nach intravenöser Injektion von sehr großen, aber nicht tödlichen Vitamin B₁-Dosen beobachtet, die sich besonders deutlich in den Ohr-

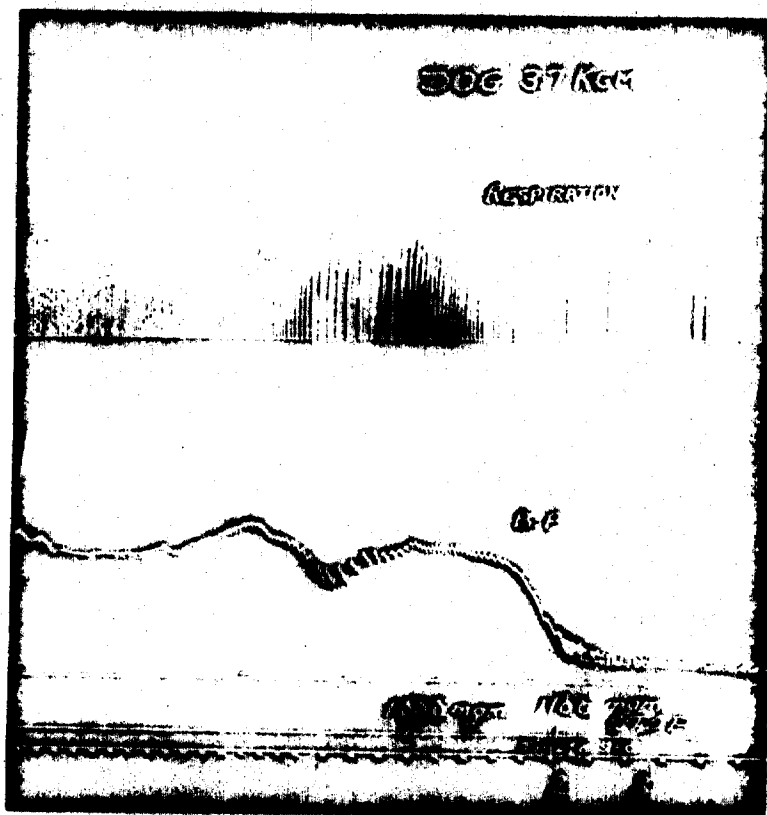


Abb. 2.

Hamd, 2,7 kg. Urethan-Narkose (1,5 g/kg).

Obere Kurve: Atmung.

Untere Kurve: Karotis-Blutdruck.

Zeitmessung alle 20 Sekunden.

Kontinuierliche Infusion einer 10%igen Lösung von Vitamin B₁ in die Hals-Jugularvene mit einer Geschwindigkeit von 0,5 cm³/Minute.

Als Marke 1: 1000 mg infundiert.

Als Marke 2: 1100 mg infundiert.

Als Marke 3: Infusion abgebrochen.

gefäßen des unbetäubten Kaninchens mittels der von Molitor u. Kniazuk¹⁰ angegebenen photo- und thermoelektrischen Methode verfolgen ließ. Abbildung 3 gibt einen Ausschnitt aus einem

solchen Versuch wieder. Wiederholte Einspritzung einer Pufferlösung von p_H 3,5 zieht keine derartige Veränderung nach sich (Abb. 4).

In einigen Versuchen hatten wir den Eindruck, daß Injektionen großer Vitamin B_1 -Dosen zu einer gesteigerten nervösen Erregbarkeit der Tiere führen. So wiesen Ratten, die sich in einer Stoffwechselapparatur nach Richards und Collison befanden, nach subkutaner Injektion großer Vitamin B_1 -Mengen zumeist einen we-

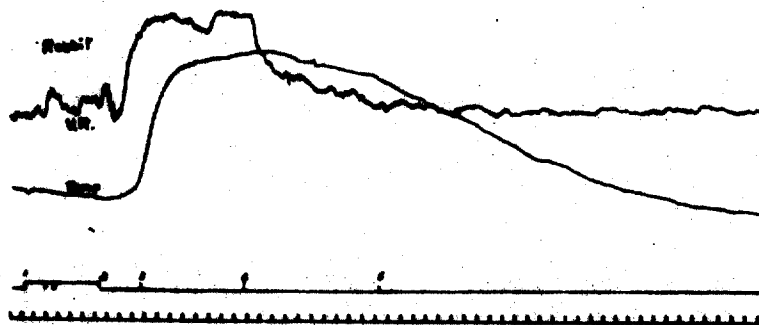


Abb. 3.

Kaninchen, 3 kg.

Photozelle und Thermoelement am linken Ohr.

Obere Kurve: (V. Z.) Änderungen der durch das Ohr durchtretenden Lichtmenge, entsprechend Änderungen der Durchblutung.

Untere Kurve: (Temp.) Änderungen der Hauttemperatur.

Zeiteinteilung alle 15 Sekunden.

Zwischen Marke 1 und Marke 3 intravenöse Injektion von 200 mg Vitamin B_1 .

Marke 3: Messung der Hauttemperatur ($33,8^\circ C$).

Marke 4: Messung der Hauttemperatur ($36,2^\circ C$).

Marke 5: Messung der Hauttemperatur ($35,9^\circ C$).

sentlich unruhigeren Kurvenverlauf auf als in dem unmittelbar vorausgegangenen Zeitabschnitt (Abb. 5). Dabei war aber der Grundniveau selbst nicht nennenswert verändert. Eine ähnliche Steigerung der Erregbarkeit fand sich auch häufig an Kaninchen, bei denen das Spiel der Ohrgefäßreflexe auf den Grad der nervösen Erregbarkeit zu schließen gestattet. Wie aus Abbildung 6 ersichtlich ist, verlaufen die Kurven der Hautdurchblutung und Hauttemperatur am normalen Kaninchen fast in einer Geraden

und zeigen nur dann eine plötzliche Änderung, wenn man starke Sinnesreize auf das Tier einwirken läßt. Nach intravenöser Injektion von 200 mg Vitamin B₁ verändert sich aber das Bild. Als Ausdruck der allgemein gesteigerten Reaktivität des Nervensystems schwanken beide Kurven, unabhängig von spezifischen Sinnesreizen, beinahe ununterbrochen auf und ab.

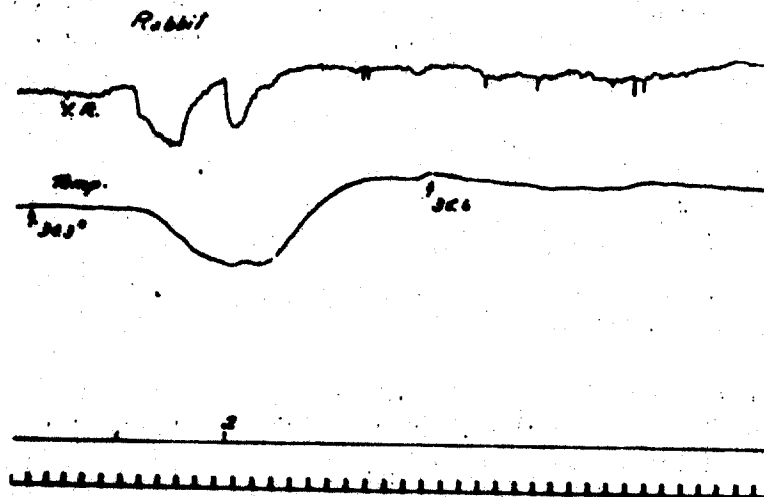


Abb. 4.

Kaninchen, 3 kg.

Photovelle und Thermoelement am linken Ohr.

Oberer Kurve: (V. R.) Änderungen der durch das Ohr durchtreten-
den Lichtmenge, entsprechend Änderungen der Durchblutung.

Untere Kurve: Änderungen der Hauttemperatur.

Zeitmarkierung alle 15 Sekunden.

Zwischen Marke 1 und Marke 2 intravenöse Injektion von 2 ccm
einer Pufferlösung von $pH = 3,5$.

Wenn auch die eben beschriebenen Veränderungen am Gefäß- und Nervensystem nicht mit solcher Regelmäßigkeit auftreten, daß wir sie als typische Begleiterscheinungen der Vitamin B₁-Überdosierung hinstellen möchten, so glauben wir doch, sie erwähnen zu sollen, zumal Kontrollversuche mit Lösungen gleicher Wasserstoffionenkonzentration negativ verliefen.

Örtliche Reizwirkungen von Vitamin B₁.

An verschiedenen Tierarten wurden die üblichen Versuche zur Prüfung örtlicher Reizwirkungen angestellt. Die zur Anwendung

gelangenden Methoden waren intrakutane Injektionen in die Bauchhaut von Meerschweinchen und zwischen die oberflächlichen Gewebeschichten des Kaninchenohres, subkutane Injektionen unter die Rückenhaut von Affen und Ratten, intramuskuläre Injektionen in die Glutäal- und Oberschenkelmuskulatur von Hunden und Einträufelungen in Kaninchen- und Katzenaugen. Die Vitamin-Lösungen waren 1, 5 und 10%ig und absichtlich nicht gepuffert, sondern auf ihrer natürlichen Wasserstoffionenkonzentration von p_H 3,5 belassen worden. In allen Fällen wurden Kontrollversuche mit einer Pufferlösung von p_H 3,5 an-

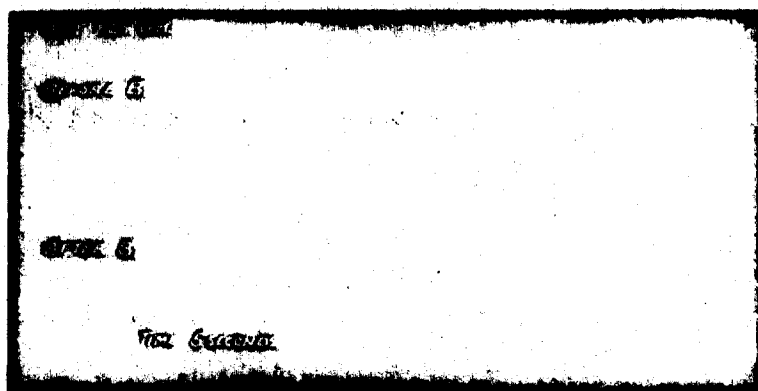


Abb. 1.

Ratte, 125 g.

Obere Kurve: Grundumsatz vor Vitamin B_1 -Injektion.

Untere Kurve: Grundumsatz nach subkutaner Injektion von 30 mg Vitamin B_1 .

Zeitmachierung alle 5 Sekunden.

Jede Zacke entspricht einem Sauerstoffverbrauch von 1,8 ccm.

gestellt, enthaltend 0,10 n Natriumchlorid, 0,10 n Aminoacetessigsäure und 0,10 n Salzsäure. Die zur Verwendung gelangenden Vitamin B_1 -Lösungen waren 30 Minuten bei 120° C im Autoklaven sterilisiert worden; biologische Auswertung der so behandelten Lösungen hatte gezeigt, daß dabei keine Einbuße an Wirksamkeit erfolgt. Die geringen, unmittelbar der intrakutanen Injektion von 0,1 ccm der betreffenden Lösungen folgenden Reizerscheinungen waren bei den Vitamin- und Kontrolllösungen gleich. Während die durch die letzteren verursachten Reizerscheinungen aber nach 24 Stunden gänzlich abgeklun-

gen waren, bestanden sie noch zu diesem Zeitpunkt bei den Vitaminlösungen und heilten nach wenigen Tagen mit Schorfbildung ab.

Subkutane Injektionen von 0,5 ccm der 1%igen, 5%igen und 10%igen Vitaminlösungen bewirkten bei Ratten eine starke örtliche Reizung, die am nächsten Tage zu Blasenbildung und ausgedehnten Epithelverlusten führte. Ähnliche, wenn auch schwä-

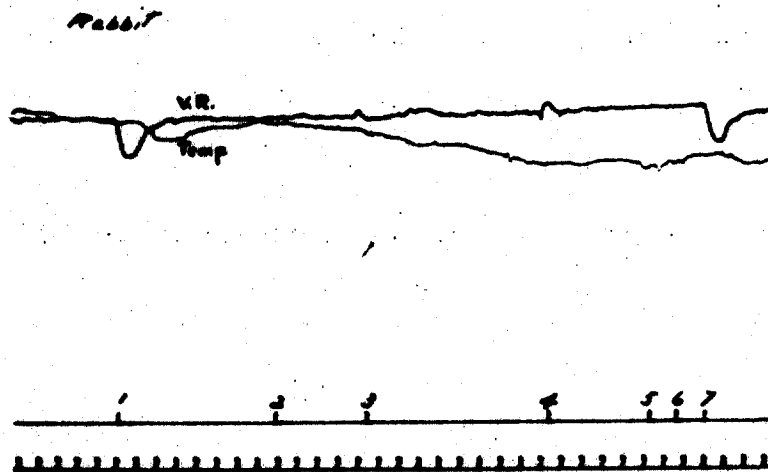


Abb. 6a.

Kaninchen, 2,8 kg.

Photocelle und Thermoelement am linken Ohr.

Oberer Kurve: (V. R.) Änderungen der durch das Ohr durchtretenden Lichtmenge, entsprechend den Änderungen der Durchblutung.

Untere Kurve: (Temp.) Änderungen der Hauttemperatur.

Zeitmachierung alle 15 Sekunden.

1—7 Geruchereize.

chere Wirkungen wurden bei Kaninchen beobachtet, während sie bei Affen und Hunden kaum sichtbar waren und sich auf eine leichte, durch 12—24 Stunden bestehende Rötung beschränkten. Intramuskuläre Injektionen wurden sowohl von Affen als auch von Hunden ohne die geringsten subjektiven oder objektiven Reizerscheinungen ertragen.

Bei keiner der verwendeten Tierarten trat Thrombosierung nach intravenöser Injektion ein.

Einträufelung von 10%igen Vitamin B₁-Lösungen in die Augen von Katzen und Kaninchen führte zu geringen Reizerscheinungen, die aber nicht stärker waren als die nach Einträufelung einer kein Vitamin enthaltenden Kontrollösung von gleicher Wasserstoffionenkonzentration.

Besprechung.

Unsere Versuche ergeben, daß reines Vitamin B₁ in außerordentlich großen Mengen akut schwere pathologische Verände-

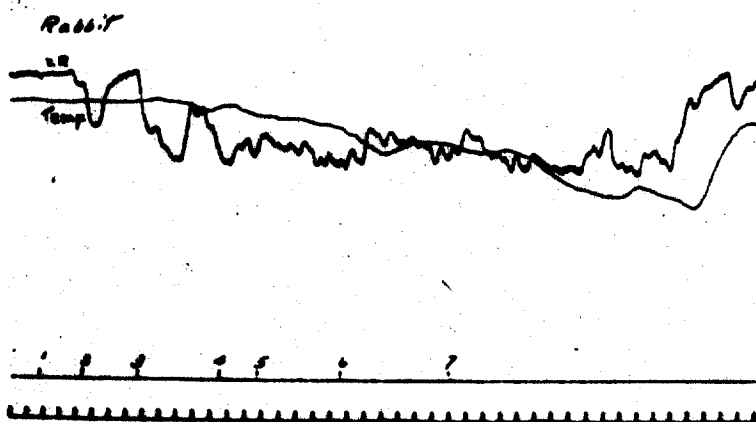


Abb. 6b.

(Nach intravenöser Injektion von 300 mg Vitamin B₁).

1. Hauttemperaturmessung (37,6° C).
2. u. 3. Öffnen der Türe des Versuchsaumes und Umhergehen.
4. Geruchswahn.
5. Hauttemperaturmessung (36,3° C).
6. Geruchswahn.

rungen und Tod herbeiführen kann. Die hierfür notwendigen Dosen sind jedoch so übertrieben groß, daß man berechtigt ist, reines Vitamin B₁ für die praktische Anwendung als völlig ungiftig anzusprechen. Wie außerordentlich weit der Abstand zwischen therapeutisch wirksamer und toxischer Dosis ist, geht aus nachfolgender Überlegung hervor, deren rechnerischer Teil auf den von Cowgill¹¹⁾ angegebenen Formeln über Vitamin B₁-Bedarf beruht. Die täglich erforderlichen Minimalmengen von Vitamin B₁ sind für Mäuse 4 Mikrogramm, für Ratten 6 Mikrogramm, für

Hunde 18 Mikrogramm. Demgegenüber betragen die intravenös tödlichen Dosen für Mäuse 2500 Mikrogramm, für Ratten 30 000 Mikrogramm und für Hunde zwischen 1 200 000 und 1 600 000 Mikrogramm. Mithin beträgt die intravenös tödliche Dosis bei Mäusen das 600fache, bei Ratten das 5000fache und bei Hunden das 67 000fache der therapeutischen. Berücksichtigt man weiter, daß die subkutan tödliche Dosis bei Mäusen und Ratten das Sechsfache und die peroral tödliche Dosis bei Mäusen das Vierzigfache der intravenös tödlichen beträgt, so ist die zuvor geäußerte Ansicht über die in praxi akute Ungiftigkeit von Vitamin B₁ wohl nicht unberechtigt. Allerdings gründet sich diese Meinung nur auf Tierversuche, die zum großen Teil an Kleintieren angestellt wurden. In diesem Zusammenhange verdient aber die Tatsache hervorgehoben zu werden, daß die Giftigkeit von Vitamin B₁ pro Einheit und Körpergewicht am größten gerade bei Kleintieren ist, während sie mit aufsteigender Tierreihe rasch abnimmt. Intravenöse Injektionen von je 1 g Vitamin B₁ in 10%iger Lösung an 2 Rhesus-Affen von 5,5 kg Gewicht mit einer Injektions-Geschwindigkeit von 2 ccm pro Minute waren von keinerlei pathologischen Erscheinungen begleitet.

Die in den vorliegenden Versuchen beschriebenen Ergebnisse beziehen sich zunächst nur auf den akuten Toxizitätsversuch und müssen für die Beurteilung eines Mittels, das vornehmlich zur Verabreichung über einen längeren Zeitraum bestimmt ist, durch die Ermittlung der Toxizität bei chronischer Darreichung ergänzt werden. Derartige Versuche werden inzwischen seit über einem Jahre durchgeführt und lassen ebenfalls schon erkennen, daß klinisch nachweisbare Schädigungen dabei nicht auftreten und das Vitamin B₁ eine außerordentliche therapeutische Breite besitzt. Über diese Versuche wird in Kürze an anderer Stelle ausführlicher berichtet werden.

Schlußsätze.

1. Die akut tödlichen Dosen von natürlichem oder synthetischem Vitamin B₁ betragen bei intravenöser Beibringung für Mäuse 125 mg/kg, für Ratten 250 mg/kg, für Kaninchen 300 mg/kg, für Hunde 350 mg/kg. Die tödlichen Dosen betragen bei subkutaner Darreichung ungefähr das Sechsfache, bei peroraler ungefähr das Vierzigfache der intravenösen.

2. Bei Verabfolgung tödlicher Vitamin B₁-Mengen geht Atemstillstand dem Herzstillstand voraus. Das Herz schlägt kräftig und regelmäßig bis zum Ende. Die äußeren Zeichen der Vergiftung sind Schock, Muskelzittern, gelegentliche klonische Krämpfe und Störungen der Atmung.

Toxizität von natürlichem und synthetischem Vitamin B₁ bei Ratten u. Mäusen.

Tierart	Material	Intravenös			Subkutan			Peroral		
		Dosis in mg	Gesamtzahl der Tiere	Davon überlebend	Dosis in mg	Gesamtzahl der Tiere	Davon überlebend	Dosis in mg	Gesamtzahl der Tiere	Davon überlebend
Maus	Synthetisch	2.5	2	2	10	4	4			
	"	2.5	3	1	12	2	2			
	"	2.75	2	0	15	2	0			
	Natürlich	2.0	6	6	10	5	5	75	3	3
	"	2.5	2	2	12	5	4	100	2	0
	"	3.0	2	0	15	4	0	120	2	0
	Gepuffert pH 6.6	2.5	2	2	10	3	3			
	"	2.5	2	0	12	4	4			
	"	3.0	2	0	15	2	0			
	"									
Ratte	Natürlich	10	3	3	100	6	6			
	"	20	6	5	120	2	2			
	"	25	3	1	150	2	2			
	"	30	2	0	180	2	1			
	"				200	3	1			
	Synthetisch	10	2	2						
	"	20	2	2						
	"	25	2	1						

3. Vitamin B₁-Lösungen lassen sich im Autoklaven bei 120° C 30 Minuten sterilisieren, ohne dabei an Wirksamkeit einzubüßen.

4. Wiederholte intravenöse und intramuskuläre Injektionen von Vitamin B₁ wurden von allen verwendeten Tierarten (Maus, Ratte, Meerschweinchen, Kaninchen, Hund, Affe) ohne die geringsten subjektiven oder objektiven Reizerscheinungen vertragen. Intrakutane und subkutane Injektionen verursachten bei Ratten und Meerschweinchen stärkere örtliche Reizerscheinungen, während solche bei Kaninchen, Hunden und Affen fehlten.

5. Reines Vitamin B₁ besitzt eine so außerordentliche therapeutische Breite, daß es für alle in Frage kommenden therapeutischen Zwecke praktisch als ungiftig zu bezeichnen ist.

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Effects of Excess Thiamine and Pyridoxine on Growth and Reproduction in Rats

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INTRODUCTION

Although studies on vitamin interrelationships have shown little or no effect of excess thiamine or pyridoxine in growing rats (Scott and Griffith, '58; Morrison and Sarett, '59), the study of Richards ('45) and the report of Hunt et al. ('54) suggest that excess amounts of either of these vitamins may have deleterious effects in pregnancy. Richards ('45) found that administration of excess thiamine to female rats fed a white flour-casein diet, simulating a low-quality human diet, had no effect on growth, but adversely affected reproduction, as evidenced by high mortality and poor growth of the young. Convulsions similar to those found in pyridoxine deficiency were observed in some of the offspring, and could be prevented by giving the dams additional pyridoxine during lactation. However, signs of other deficiencies were also observed in the young rats. Hunt et al. ('54) reported that an infant, born to a mother who had received excess pyridoxine during pregnancy, exhibited convulsions which responded to pyridoxine. They postulated that excess pyridoxine intake during pregnancy may increase the pyridoxine requirement of the infant.

The present experiment was conducted to determine whether excess amounts of thiamine or pyridoxine or both affected growth, reproductive performance and vitamin stores in female rats fed an adequate diet, and to what extent these high levels of vitamins in the mothers' diet affected weight gain and vitamin stores in the young when raised on a pyridoxine-deficient diet.

MATERIAL AND METHODS

The basal 18% casein diet used in the experiment was similar to that of Sarett

and Snipper ('54) except that sucrose was used as the carbohydrate, and ascorbic acid was omitted from the vitamin mixture. In addition, the levels of thiamine·HCl and pyridoxine·HCl were changed to 150 μ g per 100 gm of diet. These levels are slightly greater than those listed by Brown and Sturtevant ('49) as the levels required by the growing rat. Four comparable groups of 12 female weanling rats each (McCollum-Wisconsin strain) were carefully selected from 19- to 21-day-old animals weighing approximately 50 gm. The animals were individually housed in screen-bottom cages and given the basal diet (diet 1), or this diet supplemented with excess thiamine (diet 2), excess pyridoxine (diet 3), or excess thiamine and pyridoxine (diet 4). Excess thiamine and pyridoxine were added at 50 times the levels used in the basal diet.

Records were kept of the amounts of food and water consumed by each rat, and the animals were weighed individually at weekly intervals. After the animals had been on experiment for 12 weeks, they were mated with stock males of proven fertility. As soon as possible after each litter was born, the number and weight of the young were recorded. When necessary, the number of young per litter was reduced to 8 at 5 days of age.

When the young had reached 21 days of age, the dams were sacrificed (without fasting) by intraperitoneal injection of Nembutal¹ solution and the livers, kidneys and adrenals were removed and weighed. The livers of the dams fed each diet were pooled and analyzed for solids, total lipid, thiamine, riboflavin, pyridoxine, pantothenate and vitamin B₁₂. The levels of solids and total lipid were determined by

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¹ Abbott.

the method of Sarett and Jandorf ('47). Thiamine was determined by the thiochrome method (Pharmacopeia, '55), while riboflavin was assayed fluorometrically (Pharmacopeia, '55). Pyridoxine was extracted by autoclaving the samples at 121°C for 5 hours in 0.055 N HCl, according to the method of Rabinowitz and Snell ('47), and assayed microbiologically with *S. carlsbergensis*. Pantothenate was determined microbiologically with *L. plantarum* by the method of Toepfer et al. ('54), following release of bound forms of the vitamin by the double enzyme system of Novelli et al. ('49). Vitamin B₁₂ was extracted with a buffered cyanide solution according to the procedure described by Gregory ('54), and assayed using *L. leichmannii* (Pharmacopeia, '55).

At weaning (19 to 21 days of age) representative young were killed and the livers of the animals from each group were pooled (sexes kept separate) and analyzed for solids, total lipid, thiamine and pyridoxine, by the methods outlined above. The remaining animals were individually housed in screen-bottom cages and were given a diet similar to diet 1, except that pyridoxine was omitted, and the level of thiamine was increased to 200 µg per 100 gm of diet. The weight gains of these animals were followed. After two and 5 weeks, one-third of the surviving males

and females from each group were sacrificed. The livers were pooled as before and analyzed for solids, total lipid, thiamine and pyridoxine. The data on liver solids and total lipid levels in these animals showed no effect of maternal diet and are not reported herein. Most of the remaining rats died before the planned 8-week terminal analyses could be performed.

RESULTS AND DISCUSSION

Data on food and water intakes, weight gains and food efficiency values obtained before the animals were mated are summarized in table 1. In 12 weeks, the animals fed the control diet (diet 1) gained 145 gm, with an average food efficiency of 17.9 gm gain per 100 gm food intake. Addition of excess thiamine (diet 2) or excess pyridoxine (diet 3) had no significant effect on weight gain or food efficiency. However, supplementation with high levels of both thiamine and pyridoxine (diet 4) resulted in slightly greater weight gain and somewhat increased efficiency of food utilization. Although these differences were not significant, the results suggest that the thiamine and pyridoxine levels in diet 1 may have been borderline for maximum growth.

The data on reproductive performance (table 2) show that the average birth

TABLE 1
Data on weight gains, food and water intakes and food efficiencies of female rats fed diets containing various levels of thiamine and pyridoxine for 12 weeks

	Diet 1 Adequate thiamine, pyridoxine	Diet 2 Excess thiamine	Diet 3 Excess pyridoxine	Diet 4 Excess thiamine, pyridoxine
No. of survivors ¹	11	12	12	11
Initial weight, gm	49	49	49	49
8 weeks				
Weight gain, gm	106 ± 11 ²	109 ± 12	112 ± 9	116 ± 16
Food intake, gm	372	380	387	393
Water intake, ml	557	558	564	583
Food efficiency ³	28.5 ± 2.1	28.8 ± 1.8	29.0 ± 2.6	29.5 ± 2.8
12 weeks				
Weight gain, gm	145 ± 19	146 ± 15	153 ± 14	158 ± 19
Food intake, gm	811	816	827	827
Water intake, ml	1354	1702	1620	1443
Food efficiency ³	17.9 ± 1.4	18.0 ± 1.1	18.6 ± 1.8	19.2 ± 1.8

¹ Each group contained 12 animals initially.

² Standard deviation.

³ Average grams gain per 100 gm food intake.

TABLE 3
Data on reproductive performance of female rats fed diets containing various levels of thiamine and pyridoxine

No. and description of diet	No. of animals	No. of litters	No. of young	Av. birth weight of young	Survival data				Av. weight of young, 21 days
					5 days		21 days		
				gm	no.	%	no.	%	gm
1. Adequate thiamine, pyridoxine	10	6	44	6.5	43	98	32	73	37
2. Excess thiamine	12	11	75	6.3	72	98	57	76	43
3. Excess pyridoxine	11	9	75	6.1	67	89	56	75	41
4. Excess thiamine, pyridoxine	10	9	87	6.2	78	90	56	64	39

weight, survival of the young to weaning, and average weight of the young at weaning were not significantly influenced by addition of excess thiamine or pyridoxine or both to the basal diet. However, the animals which received excess thiamine and pyridoxine (diet 4) had more young per litter than those fed the other diets. The lack of any deleterious effect of excess thiamine on reproduction observed in the present study is in contrast to the findings of Richards ('45). The discrepancy in the results may be due, in part, to the relative inadequacy of the diet used by Richards, since Morrison and Sarett ('59) have found that excess amounts of a single B vitamin may retard weight gain of animals fed diets low in several B vitamins, but usually have no effect in animals fed adequate diets.

The data on organ weights of the post-parturient females showed some hypertrophy due to pregnancy, but little effect of vitamin excess. The liver weights averaged 4.6 to 5.1% of the body weight, and the adrenal glands averaged 21.3 mg to 23.5 mg per 100 gm body weight. The kidney weights of the animals on diets 2, 3 and 4 averaged 0.73 to 0.78% of body weight, whereas those of the rats on diet 1 were slightly heavier, namely 0.82% of body weight.

The levels of solids and total lipid in the livers of the post-parturient females (table 3) were not significantly influenced by addition of excess amounts of thiamine or pyridoxine or both to the basal diet. The levels of riboflavin, pantothenate and vitamin B₁₂ were also not significantly influenced by the diet fed. The addition of excess thiamine to the basal diet increased liver thiamine levels from 2.6 to 8.7 μ g per gm. In contrast to the findings with thi-

amine, the addition of excess pyridoxine to the diet had no significant effect on liver pyridoxine levels. Other workers have also found that increasing the level of thiamine in the diet above that needed for maximum growth, increases tissue concentrations of the vitamins (Ochoa and Peters, '38; Byerrum and Flokstra, '51), whereas the concentration of pyridoxine in the liver of growing rats is not influenced by addition of pyridoxine to an adequate diet (Sheppard and McHenry, '46). Excess pyridoxine apparently had no effect on liver thiamine levels, nor did excess thiamine influence the level of pyridoxine in the liver.

The data on weight gains of the young on the pyridoxine-deficient diet are summarized in table 4. The young of mothers which received excess pyridoxine (diets 3 and 4) gained significantly more weight than did those of mothers which received the basal diet. These findings do not support the hypothesis of Hunt et al. ('54) that administration of excess pyridoxine to the mother during pregnancy increases the pyridoxine dependency of the young. The presence of excess thiamine in the maternal diet (diet 2) appeared to slightly increase weight gain in the young fed a pyridoxine-deficient diet.

The data on thiamine and pyridoxine levels in the livers of the young at weaning, and after two and 5 weeks on the pyridoxine-deficient diet, are summarized in table 5. Liver thiamine levels in the young at weaning reflected maternal thiamine intakes, in a manner similar to that found in the livers of the mothers. The pyridoxine levels of the livers of the young at weaning also reflected maternal pyridoxine intake, although those in the mothers showed no effect of excess pyridoxine (table 3). At weaning, the young of ani-

TABLE 3
Data on liver composition of post-parturient female rats fed diets containing various levels of thiamine and pyridoxine

	Diet 1 Adequate thiamine, pyridoxine	Diet 2 Excess thiamine	Diet 3 Excess pyridoxine	Diet 4 Excess thiamine, pyridoxine
Number of animals per group	6	11	8	9
Solids, %	30.5	29.4	29.3	29.4
Total lipid (ether extract), %	2.7	2.7	2.4	3.3
Thiamine, $\mu\text{g/gm}$	2.6	8.7	2.3	8.4
Riboflavin, $\mu\text{g/gm}$	33.3	31.5	28.6	29.1
Pyridoxine, $\mu\text{g/gm}$	11.2	10.2	10.4	11.0
Folate, $\mu\text{g/gm}$	61.2	56.4	56.4	56.1
Vitamin B ₁₂ , mg/gm	133.5	133.8	131.7	131.7

TABLE 4
Influence of maternal diet on weight gain of male and female weanling rats fed a pyridoxine-deficient diet for 5 weeks

	Maternal diet			
	Diet 1 Adequate thiamine, pyridoxine	Diet 2 Excess thiamine	Diet 3 Excess pyridoxine	Diet 4 Excess thiamine, pyridoxine
Males				
Two weeks				
No. of animals	12	12	12	12
Weight gain, gm	11	16	31	32
Five weeks				
No. of animals	5	8	8	8
Weight gain, gm	12 ± 7^1	24 ± 8	56 ± 6	55 ± 7
Females				
Two weeks				
No. of animals	12	12	12	12
Weight gain, gm	11	14	33	30
Five weeks				
No. of animals	6	7	8	8
Weight gain, gm	14 ± 9	16 ± 7	50 ± 7	47 ± 3

¹ Standard deviation.

male fed excess thiamine (diets 2 and 4) had higher liver thiamine levels than did their mothers. However, in the animals which received excess pyridoxine (diets 3 and 4), the levels of pyridoxine in the liver were lower in the young at weaning than in the mothers, suggesting limited placental transfer of this vitamin. In man, placental transfer of vitamin B₁₂ also occurs, as shown by the work of Wachstein et al. ('57), who found that administration of pyridoxine to pregnant women resulted in elevated pyridoxal phosphate levels in the umbilical cord blood at birth. During the 5-week period on the pyridoxine-deficient diet, the greater liver vitamin

stores in the young of mothers fed excess thiamine or pyridoxine were decreased to the levels found in the young of mothers fed the basal diet.

SUMMARY

An experiment was conducted to determine whether excess thiamine or pyridoxine or both affect growth and reproduction in rats receiving an otherwise adequate diet. Groups of female weanling rats were fed a basal diet containing 150 μg of thiamine and of pyridoxine per 100 gm of diet, alone or supplemented with 50 times this level of thiamine or pyridoxine or both. After a 12-week growth period, the

TABLE 5

Influence of maternal diet on levels of thiamine and pyridoxine in the livers of male and female weanling rats fed a pyridoxine-deficient diet for 5 weeks

	Maternal diet			
	Diet 1 Adequate thiamine, pyridoxine	Diet 2 Excess thiamine	Diet 3 Excess pyridoxine	Diet 4 Excess thiamine, pyridoxine
Males				
Thiamine, $\mu\text{g/gm}$				
Initial	1.9(6) ¹	14.7(17)	1.8(9)	12.0(16)
Two weeks	2.5(4)	5.7(4)	2.6(4)	6.0(4)
Five weeks	1.6(2)	1.8(4)	2.1(4)	2.5(4)
Pyridoxine, $\mu\text{g/gm}$				
Initial	4.8(6)	5.8(17)	8.8(9)	8.7(16)
Two weeks	3.6(4)	4.0(4)	5.1(4)	5.0(4)
Five weeks	5.6(2)	4.0(4)	5.3(4)	4.8(4)
Females				
Thiamine, $\mu\text{g/gm}$				
Initial	2.0(9)	14.4(16)	1.7(15)	12.6(8)
Two weeks	2.8(4)	6.0(4)	2.7(4)	6.0(4)
Five weeks	1.6(3)	2.0(3)	2.0(4)	1.8(4)
Pyridoxine, $\mu\text{g/gm}$				
Initial	5.9(9)	5.5(16)	9.1(15)	9.7(8)
Two weeks	3.9(4)	4.0(4)	4.6(4)	5.1(4)
Five weeks	2.9(3)	3.1(3)	5.2(4)	4.2(4)

¹ Figures within parentheses indicate number of animals sacrificed to obtain pooled samples of liver.

animals were mated to stock males for study of reproductive performance. The mothers were sacrificed after the young were weaned, and liver vitamin levels were determined. The young were given a pyridoxine-deficient diet for 5 weeks, and growth and liver thiamine and pyridoxine levels were measured at intervals.

The results showed that excess thiamine or pyridoxine or both had no effect on weight gain, reproductive performance or the levels of solids, total lipid, riboflavin, pyridoxine, pantothenate and vitamin B₁₂ in the livers of the animals after parturition and lactation. Liver thiamine levels in these animals, and in their young at weaning, were markedly increased by excess thiamine. Liver pyridoxine levels in the mothers were not increased by excess dietary pyridoxine, although pyridoxine stores in the livers of the young at weaning were greatly increased by excess pyridoxine intake of the mothers.

During 5 weeks on a pyridoxine-deficient diet, the young of mothers which had received excess pyridoxine gained significant-

ly more weight than did those of mothers which had received the basal diet.

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EFFECT OF DIFFERENT QUANTITIES OF THIAMINE AND IODINE DEFICIENCY ON THE STATE OF THE THYROID GLAND IN WHITE RATS

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There is great theoretical and practical interest in the dependence of functional activity of the thyroid gland on the amount of vitamins taken into the organism. Thiamine occupies a special place among the vitamins. It is closely associated with carbohydrate metabolism, which is disrupted during thyrotoxicosis (1). Moreover, during studies conducted in the area of epidemic goiter, it was established that nutrition of the population was rich in carbohydrates (2), and this causes a significant increase in the need for thiamine. This hypothesis is borne out by the studies of V. I. Kalaykova (3), who established the noticeable increase in thiamine content in blood and urine in patients with disrupted thyroid gland function. On the other hand, several studies have established a high thiamine content in food products grown in goiter area (4), which permits the hypothesis of the presence of links between the introduction of large amounts of thiamine and the state of the thyroid gland.

The favorable effect of thiamine was noted during its administration to patients with increased thyroid gland function (5,6).

Experimental studies have established the alteration of functional state of the thyroid gland with thiamine administration (7,8).

The aim of the present study is an investigation of the effect of different amounts of thiamine in nutrition on iodine content in the thyroid glands of animals receiving an insufficient quantity of iodine.

The experiments were conducted on 133 white male rats weighing 70-120 g and placed on a starch-casein diet recommended by the Nutrition Institute, USSR Academy of Sciences. The diet consisted of starch washed of vitamins, casein, sunflower oil, and a salt mixture, from which potassium iodide was excluded. The actual content of iodine in the diet ration was 1.7 μ g/rat. The animals received with the ration 18% protein, 27% fat, and 55% carbohydrates.

The vitamins in the food had no more than the following amounts: vitamin D: 4 I.U., vitamin A: 20 I.U., riboflavin: 25 μ g, pyridoxine: 20 μ g, pantothenic acid of calcium: 150 μ g, nicotinic acid: 25 μ g, choline: 5 mg per 24 hours/rat. Thiamine was administered perorally from a pipette in 20- μ g quantities (physiological norm) and 100 μ g (experimental amount) to each rat per 24 hours. Three series of experiments were conducted for 10, 15, and 30 days. In each series the animals were divided into three groups. The rats in the 1-, 4-, and 7-day groups received 20 μ g of thiamine. The animals in the 2-, 5-, and 8-day groups received 100 μ g of thiamine. Thiamine was omitted from the food of rats in the 3-, 6-, and 9-day groups.

In the time determined in a state of deep chloroform narcosis in the animals, the thyroid glands were removed and weighed out on torsion balances. Iodine in the glands was determined according to the method of M. I. Dragomirova. The numerical data were subjected to statistical analysis with calculation of the arithmetic mean (\bar{X}), the mean standard error in the arithmetic mean ($S_{\bar{X}}$), and the level of reliability (P) according to Student's tables.

The experiments conducted revealed a real difference in thyroid gland weight in the rats, depending on the duration of the experiment and the amount of thiamine given (see table).

**Relative Weight of Thyroid Glands, Concentration, and Iodine Content in Them
in Animals Receiving a Low-Iodine Diet Containing Different Amounts of Thiamine**

Animal Group	Exper. Series	Length of Exper. (da)	# Rats in Gr.	Amt. of Thiam. in Diet	Rel. Wt. of Thyroid Gl.			I ₂ Conc. in Thyroid Gl.			I ₂ Content in Thyroid Gl.		
					X	Sy	P	X	Sy	P	X	Sy	P
					(mg)	(%)		(mg)	(%)		(mg)	(%)	
1	I	10	9	20	6.4	0.34		49.27	3.33		5.02	0.05	
2			23	100	10.3	1.02	99.9	92.25	15.25	99.9	99.71	0.02	99.9
3			10	-	10.5	0.18	99.9	35.58	9.30	78.9	6.04	1.61	44.4
4	II	15	15	20	7.2	0.53		152.74	10.55		16.27	0.41	
5			14	100	12.5	0.66	99.9	66.92	7.59	99.9	17.03	1.83	31.1
6			9	-	8.7	1.00	83.8	91.90	36.47	85.0	12.27	1.75	97.6
7	III	30	17	20	6.4	0.58		63.50	2.88		6.20	1.71	
8			16	100	10.9	0.72	99.9	41.18	5.61	99.8	8.43	1.08	72.9
9			20	-	7.6	0.48	89.0	32.56	5.99	99.9	6.60	0.81	-

As is evident from the data in the table, on the 10th day of the experiment (series I), the weight of the thyroid glands of the experimental animals in the different groups changed relative to the amount of thiamine introduced. Thus, the relative weight of the thyroid glands of rats which received physiological amounts of thiamine (1st group), on the 10th day, proved to be very low. The relative weight of thyroid glands in rats which received a surplus of thiamine (2nd group) and in rats which received an avitaminose diet (3rd group) was, on the 10th day of the experiment, higher than in animals which received the physiological amount of the vitamin.

After holding the animals 15 days on the low-iodine diet (series II of the experiments), the weight of the thyroid glands in rats in groups which received 20 and 100 μ g of thiamine was significantly increased, while the most noticeable increase in weight took place in the group of animals which received 100 μ g of thiamine. The relative weight of the thyroid glands in rats on the avitaminose diet was somewhat decreased on the 15th day of the experiment in comparison to gland weight of animals which received a physiological quantity of thiamine (4th group).

In rats held for 30 days on a low-iodine diet (series III of the experiments), thyroid gland weight proved to be the largest in the group of animals which received 100 μ g of thiamine (8th group). Thyroid gland weight in rats on the avitaminose diet was somewhat greater (by a non-significant amount) in comparison to the relative weight of animals which received a physiological dose of thiamine (7th group).

It is necessary to note that gland weight in rats which received thiamine was almost equal on the 30th day of the experiment to the gland weight on the 10th day (1st and 2nd groups).

Iodine concentration in the thyroid glands on the 10th day of the experiment proved to be the greatest in the group of animals which received an excess amount of thiamine (2nd group); iodine content in the glands of animals in this group was also the largest. The thyroid glands of animals which received a physiological amount of thiamine was distinguished by a higher ability to concentrate iodine. However iodine content in the glands of rats which received a physiological quantity of thiamine and rats on the avitaminose diet was the same.

On the 15th day of the experiment, iodine concentration in the thyroid glands of animals which received a physiological amount of thiamine (4th group) and those which received no thiamine (6th group) increased significantly in comparison to data obtained on the 10th day of the study. Specially noticeable was the increase in concentrating ability of the glands in the group of rats which received a physiological amount of thiamine (4th group). However, if you compare iodine content in the thyroid glands on the 15th day of the experiment, it appears that in the glands of animals which received thiamine (4th and 5th groups), iodine content increases significantly in comparison to its content in the glands of rats which were on the avitaminose diet (6th group).

In series III of the experiment (after 30 days), the iodine-concentrating ability of the thyroid glands was significantly decreased in all the groups of animals. In the thyroid glands of animals which received a physiological amount of thiamine in their feed (7th group), iodine concentration was the greatest and significantly greater than on the 10th day of the experiment (11th group) (sic). The concentrating ability of the thyroid gland of animals which received an excess of thiamine (8th group) was lower in comparison with iodine concentration in thyroid glands of animals which received a physiological amount of thiamine (7th group). The lowest was the level of iodine concentration in the thyroid glands of rats on the avitaminose diet (9th group). Iodine content in thyroid glands of animals on the 10th day of the experiment turned out to be almost identical. An exception was the animals which received an excess of thiamine (in their glands, iodine content was somewhat greater; $P = 72.9\%$).

As is evident from the data presented, a non-significant introduction of iodine even on the 10th day of the experiment evoked changes on the part of the thyroid gland. In addition, the reaction of the gland differed depending on thiamine content in the feed. It was more obvious with excess and deficient thiamine in the feed than in giving the animals a physiological quantity of the vitamin.

In administering a physiological dose of thiamine, the thyroid gland reacted on the 15th day of the experiment with an insignificant hypertrophy and a significant retention of iodine. On the 30th day of the experiment, the concentration and content of iodine decreased in the glands of animals which received a physiological amount of thiamine.

Excess administration of thiamine contributed to iodine accumulation in the thyroid gland on the 10th day of the experiment; in the last days of the experiment, iodine concentration in the thyroid gland was lower, and on the 15th day, there was observed no occurrence of iodine retention in the gland. However, the effect of excess thiamine was accompanied by an obvious hypertrophy of the thyroid gland. Similar results were obtained by S. M. Maksimov, Z. V. Novokhataya, and I. I. Sharkevich (9), who established that long-term administration of thiamine leads to hypertrophy of the thyroid gland and to an acceleration of the processes of radioactive iodine accumulation and its removal.

The thyroid glands of rats on the avitaminose diet under conditions of iodine deficiency reacted at first with obvious hypertrophy and later, with hypotrophy. On the 15th day of the experiment, these rats exhibited a significant iodine retention in the thyroid gland, and on the 30th day, iodine concentration in the glands decreased.

Conclusions

1. Excess doses of thiamine, administered to animals which received a low-iodine diet, caused a moderate hypertrophy of the thyroid gland and an increase in its iodine content. An especially obvious increase in iodine concentration in the animals' glands was observed in the first days of large dosage administration of thiamine to the animals.

2. Administration of physiological quantities of thiamine hindered the occurrence of hypertrophy of the thyroid gland; however, there occurred no significant effect on the increase in iodine content in them.

3. B₁ avitaminosis leads to hypertrophy of the thyroid gland and is accompanied by a decrease in the ability of the thyroid gland to concentrate iodine.

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ВЛИЯНИЕ РАЗЛИЧНЫХ КОЛИЧЕСТВ ТИАМИНА И ЙОДНОЙ НЕДОСТАТОЧНОСТИ НА СОСТОЯНИЕ ЩИТОВИДНОЙ ЖЕЛЕЗЫ БЕЛЫХ КРЫС

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Большой теоретический и практический интерес представляет зависимость функциональной активности щитовидной железы от количества витаминов, поступающих в организм. Особое место среди ряда витаминов занимает тиамин. Он тесно связан с обменом углеводов, нарушающимся при тирозинемиях [1]. Кроме того, при исследованиях, проведенных в очагах эндемической зобной болезни, установлено, что питание населения богато углеводами [2], а это вызывает значительное увеличение потребности в тиаминах. Это предположение подтверждают исследования В. И. Калмыковой [3], установившей заметное уменьшение содержания тиамина в крови и моче у больных с нарушениями функции щитовидной железы. С другой стороны, некоторыми исследованиями установлено высокое содержание тиамина в пищевых продуктах, выращенных в зобных очагах [4], что позволяет сделать предположение о наличии связи между введенным большими количествами тиамина и состоянием щитовидной железы.

Отмечено благоприятное влияние тиамина при его введении больным с повышенной функцией щитовидной железы [5, 6].

Экспериментальными исследованиями установлено изменение функционального состояния щитовидной железы при введении тиамина [7, 8].

Цель настоящего исследования состояла в изучении влияния различных количеств тиамина в питании на содержание йода в щитовидной железе животных, получающих недостаточное количество йода.

Опыты проводили на 133 белых крысах-самцах весом 70—120 г, содержащихся на крахмально-казеиновой диете, рекомендованной Институтом питания АМН СССР. В состав диеты входил крахмал, отмытый от витаминной казеин, подсолнечное масло и солевая смесь, из которой исключали йодистый калий. Естественное содержание йода в дневном рационе составляло 1,7 мкг на крысу. Животные получали с рационом 18% белка, 27% жира и 55% углеводов.

Витамины добавляли в пищу в следующих количествах: витамин D—4 МЕ, витамин A—20 МЕ, рибофлавин—25 мкг, пиридоксин—20 мкг, пантотеновокислый кальций—150 мкг, никотиновая кислота—25 мкг, холин—5 мг в сутки на крысу. Тиамин вводили перорально из пипетки в количестве 20 мкг (физиологическая норма) и 100 мкг (избыточные количества) на крысу в сутки. Проведено 3 серии опытов—10, 15 и 30 дней. В каждой серии животные были разделены на 3 группы. Крысы 1, 4 и 7-й группы получали 20 мкг тиамина. Животные 2, 5 и 8-й группы получали 100 мкг тиамина. В корме крыс 3, 6 и 9-й группы тиамина отсутствовал.

В определенные сроки в состоянии глубокого хлороформного наркоза у животных выделяли щитовидные железы и взвешивали на торсионных весах. Йод в железах определяли по методу М. И. Драгомировой. Цифровые данные подвергали статистической обработке с вычислением средней арифметической (\bar{X}) средней стандартной ошибки среднего арифметического ($S_{\bar{X}}$) и степени достоверности (P) по таблицам Стьюдента.

Проведенные исследования выявили существенную разницу в весе щитовидных желез крыс-самцов в зависимости от длительности опыта и количества вводимого тиамина (см. таблицу).

Относительный вес щитовидных желез, концентрация и содержание в них йода у животных, получающих малоiodную диету, содержащую различные количества тиамина

Группа животных	Серия опытов	Продолжительность опыта (в днях)	Число крыс в группе	Количество тиамина в диете (в мкг)	Относительный вес щитовидных желез			Концентрация йода в щитовидных железах			Содержание йода в щитовидных железах		
					\bar{X}	$S_{\bar{X}}$	ρ	\bar{X}	$S_{\bar{X}}$	ρ	\bar{X}	$S_{\bar{X}}$	ρ
					(в мг%)			(в мкг%)			(в мкг%)		
1-я	I	10	9	20	6.4	0.34		49.27	3.33		5.02	0.05	
2-я		10	23	100	10.3	1.02	99.9	92.25	15.25		99.71	0.02	
3-я		10	10	—	10.5	0.18	99.9	35.58	9.30		6.04	1.64	44.4
4-я	II	15	15	20	7.2	0.53		152.74	10.55		16.27	0.41	
5-я		15	14	100	12.5	0.86	99.9	66.92	7.59	99.9	17.03	1.83	31.1
6-я		15	9	—	8.7	1.00	83.8	91.90	36.47	85.0	12.27	1.75	97.6
7-я	III	30	17	20	6.4	0.58		63.50	2.88		6.20	1.71	
8-я		30	16	100	10.9	0.72	99.9	41.18	5.61	99.8	8.43	1.08	
9-я		30	20	—	7.6	0.48	89.0	32.56	5.99	99.9	6.60	0.81	72.9

Как видно из данных таблицы, уже на 10-й день опыта (I серия) вес щитовидных желез подопытных животных разных групп изменялся в зависимости от количества вводимого тиамина. Так, относительный вес щитовидных желез крыс, получавших физиологические количества тиамина (1-я группа), на 10-й день оказался самым низким. Относительный вес щитовидных желез животных, получавших избыточные количества тиамина (2-я группа), и крыс, находившихся на авитаминозной диете (3-я группа), на 10-й день опыта был выше, чем у животных, получавших физиологические количества тиамина.

После 15-дневного содержания животных на малоiodной диете (II серия опытов) вес щитовидных желез крыс в группах, получающих 20 и 100 мкг тиамина, значительно увеличился, причем наиболее заметное увеличение веса имело место в группе животных, получающих 100 мкг тиамина. Относительный вес щитовидных желез крыс, находившихся на авитаминозной диете, на 15-й день опыта немного уменьшился по сравнению с весом желез животных, находившихся в опыте 10 дней (3-я группа), и приближался к весу желез крыс, получавших физиологические количества тиамина (4-я группа).

У крыс, содержавшихся в течение 30 дней на малоiodной диете (III серия опытов), вес щитовидных желез оказался самым большим в группе животных, получавших 100 мкг тиамина (8-я группа). Вес щитовидных желез крыс, находившихся на авитаминозной диете, был немного больше (на недостоверную величину) по сравнению с относительным весом животных, получавших физиологические дозы тиамина (7-я группа).

Необходимо отметить, что вес желез крыс, получавших тиамин на 30-й день опыта, был почти равен весу желез на 10-й день опыта (1-я и 2-я группы).

Концентрация йода в щитовидных железах на 10-й день опыта оказалась самой большой в группе животных, получавших избыточные количества тиамина (2-я группа); содержание йода в железах животных этой группы было также самым большим. Щитовидные железы живот-

ных, получавших физиологические количества тиамина, отличались более высокой способностью концентрировать йод. Однако содержание йода в железах крыс, получавших физиологические количества тиамина, и в железах животных, находившихся на авитаминозной диете, было одинаковым.

На 15-й день опыта концентрация йода в щитовидных железах животных, получавших физиологические количества тиамина (4-я группа) и не получавших тиамина (6-я группа), значительно возросла по сравнению с данными, полученными на 10-й день исследования. Особенно заметно увеличение концентрационной способности желез в группе крыс, получавших физиологические количества тиамина (4-я группа). Однако если сравнить содержание йода в щитовидных железах на 15-й день опыта, выявляется, что в железах животных, получавших тиамин (4-я и 5-я группа), содержание йода значительно возрастает по сравнению с содержанием его в железах крыс, находившихся на авитаминозной диете (6-я группа).

В III серии опытов (спустя 30 дней) подконцентрационная способность щитовидных желез значительно уменьшилась во всех группах животных. В щитовидных железах животных, получавших с пищей физиологические количества тиамина (7-я группа), концентрация йода была самая большая и значительно больше, чем на 10-й день опыта (11-я группа). Концентрационная способность щитовидной железы животных, получавших избыточные количества тиамина (8-я группа), была ниже, по сравнению с концентрацией йода в железах животных, получавших физиологические количества тиамина (7-я группа). Самым низким оказался уровень концентрации йода в щитовидных железах крыс, получавших авитаминозную диету (9-я группа). Содержание йода в щитовидных железах животных на 30-й день опыта оказалось почти одинаковым. Исключение составили животные, получавшие избыточные количества тиамина (в их железах содержалось йода немного больше; $P=72,9\%$).

Как видно из приведенных данных, недостаточное введение йода уже на 10-й день опыта вызывало изменения со стороны щитовидной железы. При этом реакция железы была различной в зависимости от содержания тиамина в пище. Она была более выражена при избытке тиамина в пище и недостатке его, чем при даче животным физиологических количеств тиамина.

При введении физиологических доз тиамина щитовидная железа на 15-й день опыта реагировала незначительной гипертрофией и значительной задержкой йода. На 30-й день опыта уменьшались концентрация и содержание йода в железах животных, получавших физиологические количества тиамина.

Избыточное введение тиамина способствовало накоплению йода щитовидной железой на 10-й день опыта, в поздние сроки опыта концентрация йода в щитовидных железах становилась ниже, и на 15-й день опыта не наблюдалось явлений задерживания йода в железе. Однако действие избытка тиамина сопровождалось выраженной гипертрофией щитовидной железы. Подобные результаты получили С. М. Магсимов, З. В. Новохатская и И. Н. Шаркевич [9], установившие, что длительное введение тиамина приводит к гипертрофии щитовидной железы и к ускорению процессов накопления радиоактивного йода и выведения его.

Щитовидная железа крыс, находившихся на авитаминозной диете в условиях йодной недостаточности реагировала вначале выраженной гипертрофией, а в более поздние сроки — гипотрофией. На 15-й день опыта у этих крыс наблюдалась значительная задержка йода щитовидной железой, а на 30-й день опыта концентрация йода в железах уменьшалась.

Выводы

1. Избыточные дозы тиаминна, введенные животным, получавшим малоiodную диету, вызвали умеренную гипертрофию щитовидной железы и увеличение содержания йода в ней. Особенно выраженное увеличение концентрации йода в железах животных наблюдалось в первые дни введения животным больших доз тиаминна.

2. Введение физиологических количеств тиаминна препятствовало возникновению гипертрофии щитовидных желез, однако не оказывало существенного влияния на увеличение содержания йода в них.

3. Авитаминоз В₁ приводит к гипертрофии щитовидной железы и сопровождается понижением способности щитовидной железы концентрировать йод.

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THE EFFECTS OF DIVERSE AMOUNTS OF THIAMINE AND OF IODINE DEFICIENCY ON THE THYROID GLAND IN ALBINO RATS, X

I. O. Nagirna (Lvov)

Summary

The paper reports results consecutive to the study into the influence exerted by various amounts of thiamine upon the weight of the thyroid glands, the iodine content and concentration therein, as seen against the background of feeding male albino rats on a diet containing deficient amounts of iodine.

The excessive supply of thiamine was found to cause a moderate hypertrophy of the thyroid and an increased iodine content in it. In the initial period marking the action of large amounts of thiamine one can see a rising iodine concentration in the thyroid gland of experimental animals.

The administration of physiological amounts of thiamine prevents the development of thyroid hypertrophy, but fails to appreciably affect the mounting iodine level therein. Vitamin B₁ deficiency leads to hypertrophy of the thyroid gland and to a decline of the iodine concentration therein.

Glyoxylic Acid Oxidation by Rat Liver*

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The enzymatic formation of glyoxylate from tricarboxylic acids has focused attention on this α -keto acid as a possible intermediate of alternate metabolic pathways (1-3). Although the isocitrate reaction has not been shown in animal tissues, recent work has indicated that the principal degradative pathway for glycine by rat liver preparations proceeds via glyoxylic acid (4). Although the oxidation of glyoxylate to oxalate can be catalyzed by xanthine oxidase (5, 6), previous studies have shown that glyoxylate, when present in amounts expected under physiological conditions, was converted to formate and CO_2 (5). Nonenzymatically, this oxidative decarboxylation occurs quite rapidly in the presence of hydrogen peroxide and certain heavy metal ions (7). The metabolism of glyoxylate via citrate formation (1, 3) or via malate formation has been reported to occur in microorganisms and plants (3, 8). The importance of these pathways to the metabolism of mammalian tissues has not yet been assessed. This communication presents studies on the partially purified enzyme system from rat liver mitochondria that catalyzes the oxidative decarboxylation of glyoxylate to formate and CO_2 .

EXPERIMENTAL

Materials— C^{14} -labeled glyoxylic acid was synthesized from oxalic acid-1,2- C^{14} by the procedure of Weinhouse and Friedmann (9). γ -Aminoglutaric acid was synthesized by the method of Tabor and Mehler (10). All other materials were obtained from commercial sources.

Experiments with Rat Liver Homogenate—Experiments with washed homogenates of liver were carried out essentially according to procedures described by Lehninger and Kennedy (11) except that centrifugations were carried out at $6000 \times g$ for 10 minutes and the suspending medium was as described below, with normal adult rats (Wistar, Sprague-Dawley, or Slonaker). Homogenizations, washings, and incubations were carried out in isotonic KCl (70 parts), MgSO_4 (2.5 parts) and phosphate buffer, pH 7.4 (15 parts). Incubations were conducted in Warburg vessels at 37° for 1 hour with an air atmosphere. The reaction was stopped by tipping 0.2 ml. of 25 per cent trichloroacetic acid from the side arm, and CO_2 was collected on alkali-soaked filter papers and precipitated as BaCO_3 for radioactive assay.

Measurement of Radioactivity—Radioactivity was measured in a proportional, windowless, gas flow counter. The counts were measured as BaCO_3 at infinite thickness. In order to facilitate enzyme assays, a rapid method for radioactive measurements was used. 0.6 mmoles of Na_2CO_3 carrier was added to

each mmole of respired CO_2 . The average weight of BaCO_3 by this method was 118 ± 2 mg. with a spread from 116 to 123 mg. For rapid assays the respired CO_2 was collected, 1 ml. of 0.6 M Na_2CO_3 was added, followed by excess BaCl_2 . The precipitated BaCO_3 was filtered through sintered glass funnels, washed, then rapidly dried by washing with alcohol and 1:1 acetone-petroleum ether. The samples were placed under a heat lamp for a few minutes and then were ready for plating and counting. The average weight of 118 mg. of BaCO_3 was assigned to these samples. The time lapse from the end of incubation to counting chamber could be reduced to as little as 15 minutes.

Formate—Approximately 1 mmole of carrier was added to the deproteinized solution; formate was steam-distilled, concentrated, and oxidized with mercuric ions, as already described (12).

Glycine—Carrier glycine was added to the deproteinized solution and after formate had been removed by steam distillation, sufficient BaCl_2 was added to remove sulfate ions. After removal of BaSO_4 and decolorization, the solution was transferred to a Dowex 50 column and chromatographed by the method of Hirs, Moore, and Stein (13).

Paper Chromatography—Paper chromatograms were developed with 80 per cent phenol-water or *tert*-amyl alcohol, formic acid, and water (70:15:15). Amino acids were detected with ninhydrin and *N*-formylglutamic acid with Cl_2 , KI, and starch (14).

Glyoxylate Metabolism by Washed Homogenates of Rat Liver—Preliminary studies on glyoxylate oxidation by rat liver homogenates showed a variable but significant rate of breakdown. A clue to a possible mechanism for glyoxylate catabolism was provided during studies on glycine formation by transamination. Nonenzymatic transamination can occur between glyoxylate and a number of amino acids (15). In testing for enzymatic glycine formation, washed homogenates of rat liver were incubated with C^{14} -labeled glyoxylate in the presence or absence of *L*-glutamate (Table I). The added *L*-glutamate not only increased glycine formation 50- to 70-fold, but also increased production of radioactive CO_2 approximately 15-fold. This stimulating effect of *L*-glutamate on glycine formation and CO_2 production was essentially identical under either aerobic or anaerobic conditions. Formate accumulation closely paralleled CO_2 production. It is apparent from these results that the products of glyoxylate metabolism in this preparation were formate and CO_2 . Formaldehyde formation could not be shown in this or in similar preparations. An attempt was made to stimulate glycine metabolism with α -ketoglutarate or *L*-glutamate. Rather than stimulate glycine metabolism, α -ketoglutarate or *L*-glutamate appeared to inhibit the reaction.

Weinhouse and Friedmann (9) showed the rapid oxidation of glyoxylate by the intact rat. Table II gives the results of a survey of various tissues of the rat for the ability to oxidize

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glyoxylate. Liver, kidney, and heart were most active but in contrast with the glyoxalic acid dehydrogenase system (5), other tissues also contained the glyoxalic acid dehydrogenase system in varying but nonnegligible amounts. This wide distribution of glyoxylate oxidation suggests that there may be some source of glyoxylate other than glycine in animals, or that this system is not specific for glyoxylate, but capable of oxidizing other substrates. Comparison of rat liver homogenates, fractionated by differential centrifugation according to the method of Hogeboom (16), showed that glyoxylate oxidation was catalyzed by the mitochondrial particles.

Partial Purification of Glyoxylate Oxidizing System—Mitochondria from approximately 100 gm. of rat liver were obtained by the sucrose fractionation method of Hogeboom (16). The mitochondria were extracted with 100 ml. of 0.01 M phosphate buffer, pH 7.4, by blending a suspension of the mitochondria and 10 gm. of powdered glass in an ice-jacketed Waring Blender for 10 minutes. The temperature was maintained below 5° by alternate periods of blending and cooling. Unless otherwise stated, all subsequent operations were carried out at 0–5°. The suspension was centrifuged at 18,000 × *g* for 20 minutes. The slightly opaque, light red supernatant fluid was diluted to 200 ml. with water, and 78 gm. of ammonium sulfate (0 to 60 per cent saturation) were added. The precipitate obtained on centrifugation was dissolved in 200 ml. of water and treated with ammonium sulfate in order to obtain fractions of 0 to 30 per cent, 30 to 40 per cent, and 40 to 50 per cent saturated. The 30 to 40 per cent and 40 to 50 per cent fractions were dissolved in 25 ml. of 0.02 M phosphate buffer, pH 7.0, and dialyzed against this buffer overnight. The dialyzed fractions were treated with 2 ml. of calcium phosphate gel (50 mg./ml.), stirred, and centrifuged. The resulting clear, pale yellow solutions contained the glyoxalic acid dehydrogenase system, the 30 to 40 per cent saturated fraction generally having the greater activity. The protein concentration of this fraction, determined by the method of Warburg and Christian (17), generally was 3 to 7 mg. of protein per ml. and represented a purification of approximately 5- to 10-fold over the original mitochondrial extracts.

This preparation was stable at refrigerator temperatures for at least 1 week and could be stored for longer periods in the frozen state with about 25 per cent loss in activity for each freeze-thaw operation. Heating the enzyme at 50° for 5 minutes, or acidifying to pH 5.5 with acetic acid at 0°, rapidly inactivated the enzyme system.

Properties of Enzyme System—The preparation described was contaminated with other enzymes. L-Glutamic acid dehydrogenase and lactic acid dehydrogenase activities were found in every preparation. Since lactic acid dehydrogenase and DPN¹ have been shown to catalyze the interconversion of glyoxylate and glycolate (5), this enzyme probably aids glyoxylate oxidation by regenerating DPN from the DPNH formed from the action of either or both the glyoxalic acid dehydrogenase or L-glutamic acid dehydrogenase. These contaminants have far prevented the application of a spectrophotometric assay of DPN reduction to the study of this enzyme system. That neither of these two contaminating enzymes was directly involved in glyoxylate oxidation was shown by heating the

TABLE I
Transamination and oxidation of glyoxylate by washed homogenate of rat liver

Substrates	Gas phase	Respired CO ₂	Glycine	Formate
		Micromoles C ¹⁴ /gm. tissue/hr.		
Glyoxylate-1,2-C ¹⁴	Air	0.5	0.7	0.4
Glyoxylate-1,2-C ¹⁴ + L-glutamate.....	Air	6.5	18.2	4.5
Glyoxylate-1,2-C ¹⁴	N ₂	0.4	0.3	0.4
Glyoxylate-1,2-C ¹⁴ + L-glutamate.....	N ₂	5.8	18.5	4.1

Each flask contained washed homogenate of rat liver (from 330 mg. of liver) suspended in KCl, MgSO₄, phosphate buffer at pH 7.4, and substrates as indicated. The concentrations were: glyoxylate-1,2-C¹⁴, 0.005 M, and glutamate, 0.01 M. Flasks were incubated for 1 hour at 37°.

TABLE II
Glyoxylate oxidation by various rat tissues

Organ	Respired CO ₂
	μmoles
Liver.....	10.4
Kidney.....	8.7
Heart.....	6.8
Muscle.....	3.5
Lung.....	2.5
Brain.....	2.5
Spleen.....	1.6

Each flask contained washed homogenate of rat liver (from 330 mg. of liver) suspended in KCl, MgSO₄, phosphate buffer, at pH 7.4, glyoxylate-1,2-C¹⁴, 0.005 M, and L-glutamate, 0.01 M. The total volume was 3 ml. Flasks were incubated for 1 hour at 37°.

enzyme preparation at 50° for 5 minutes. Under these conditions lactic acid and L-glutamic acid dehydrogenase activities were retained while glyoxalic acid dehydrogenase was completely destroyed.

The pH optimum for this system (Fig. 1) ranges from pH 6.7 to 7.

Requirements for Glyoxylate Dehydrogenase System—Early experiments indicated that glutamate and DPN were necessary for activity. The system that appears to be necessary for maximum activity is shown in Table III. In addition to L-glutamate and DPN, Mg⁺⁺, Ca⁺⁺, or Mn⁺⁺ ions and thiamine pyrophosphate were found to enhance glyoxylate decarboxylation. With this enzyme preparation there appears to be some bound DPN present, as shown by the residual activity when DPN was omitted from the reaction mixture. This may explain the slight enhancement of activity when DPN was replaced by TPN, but the results suggested that this system was specific for DPN. An absolute requirement for Mg⁺⁺, Ca⁺⁺, or Mn⁺⁺ ions could not be shown, but any one of these ions stimulated CO₂ formation from glyoxylate, the latter giving the greater stimulation. The fact that thiamine pyrophosphate stimulated the reaction, coupled with the further enhancement noted with DPN and the metal ions, suggests that the mechanism for glyoxylate catabolism

¹ The abbreviations used are: DPN, and DPNH, oxidized and reduced forms of diphosphopyridine nucleotide; TPN, triphosphopyridine nucleotide.

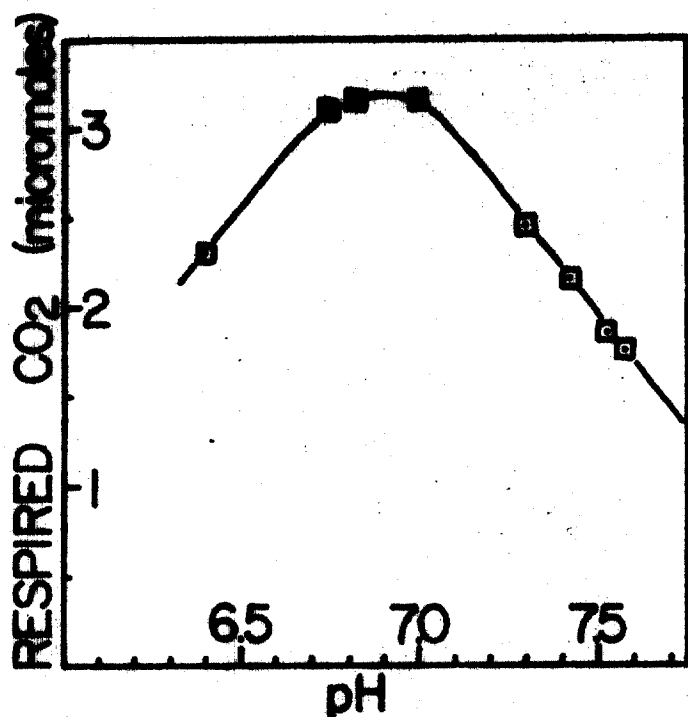


Fig. 1. Effect of pH on glyoxylic acid dehydrogenase. Each flask contained the components for the complete system (Table III) with the exception that phosphate buffers of varying pH values were used. The pH values were determined at the end of the incubation period.

TABLE III
Requirements for maximal activity

System	Respired CO ₂ (per cent maximal activity)
Complete system	100
Complete system minus L-glutamate	4.8
Complete system minus DPN	21
Complete system with TPN replacing DPN	35
Complete system minus thiamine pyrophosphate	48
Complete system minus MnCl ₂	57
Complete system with MgCl ₂ replacing MnCl ₂	84
Complete system with CaCl ₂ replacing MnCl ₂	90

Complete system: Enzyme (1 ml.), glyoxylate-1,2-C¹⁴ (5 μ moles 131,000 c.p.m. as BaCO₃ at infinite thickness), L-glutamate (5 μ moles), MnCl₂ (8 μ moles), DPN (2.7 μ moles), thiamine pyrophosphate (1.2 μ moles), 0.1 M phosphate buffer, pH 6.8 (0.7 ml.), and H₂O to 2.5 ml. total volume. 0.2 ml. of 5 N NaOH and a small square of filter paper were placed in the center well to trap CO₂, and 0.2 ml. of 25 per cent trichloroacetic acid was placed in the side arms. The trichloroacetic acid was tipped from the side arm to terminate enzyme action. The flasks were incubated at 37° for 1 hour.

can be compared with the oxidative decarboxylation of other α -keto acids such as pyruvate or α -ketoglutarate. Other co-factors such as coenzyme A, adenosine triphosphate, adenosine diphosphate, folic acid, pyridoxal phosphate, pyridoxamine phosphate, and glutathione were tested and found to be inactive. DL-Thioctic acid occasionally produced a slight and variable

TABLE IV
Effect of glutamate concentration on glyoxylate oxidation

Glutamate concentration μ moles/2.5 ml.	Respired CO ₂ (c.p.m. per 150 mg. BaCO ₃ per flask)
0	24
0.1	24
0.5	240
1.0	317
5	397
10	301
20	296
30	216

Each flask contained glyoxylate-1,2-C¹⁴ (5 μ moles 131,000 c.p.m. as BaCO₃ at infinite thickness), enzyme (1 ml.), L-glutamate in amounts indicated, DPN (2.7 μ moles), thiamine pyrophosphate (1.2 μ moles), MnCl₂ (8 μ moles), phosphate buffer, pH 6.8 (70 μ moles), and H₂O to 2.5 ml. total volume. Incubations were carried out at 37° for 1 hour.

stimulation in the glyoxylic acid dehydrogenase system. This was not considered sufficiently significant to include as a requirement for the system.

The complete system generally used for all of the experiments is outlined in the legend for Table III.

Effect of L-Glutamate Concentration—A series of experiments was set up, holding the concentration of all components of the system except L-glutamate constant. Typical results from such experiments (Table IV) showed that small amounts of L-glutamate stimulated glyoxylate oxidation but the optimum effect was found to be at equimolar concentrations of glyoxylate and L-glutamate. Higher L-glutamate concentrations were inhibitory. When the ratio of glyoxylate and L-glutamate concentrations was held constant at 1, and the amounts were increased, the rate of glyoxylate oxidation reached a plateau at an approximate substrate concentration of 0.005 M. These results indicate that L-glutamate was not acting catalytically. The stoichiometry suggests that a condensation may have occurred between glyoxylate and L-glutamate.

Specificity of L-Glutamate—The data presented (Table III) showed that L-glutamate was absolutely required for the glyoxylic acid dehydrogenase system. Table V summarizes the results of a study designed to demonstrate the specificity of L-glutamate for the activation of the oxidative decarboxylation of glyoxylate. L-Glutamine was about one-half as effective as L-glutamate. As illustrated in Table IV, trace quantities of L-glutamate could increase the rate of glyoxylate decarboxylation. It is not known whether the increase observed in the presence of L-glutamine was caused by a slight conversion of the amide to the free acid or whether the increase was caused by L-glutamine itself. It is to be noted that the unnatural isomer, D-glutamate, as well as other close analogues of L-glutamate, such as N-formylglutamate, α -methylglutamate, γ -aminobutyrate, and L-aspartate, could not replace L-glutamate in this system. Other amino compounds tested could not activate this system significantly. Specificity of this type suggests that L-glutamate is involved in the glyoxylic acid dehydrogenase system at the enzyme level.

Effect of Inhibitors—Several typical enzyme inhibitors were tested (Table VI). Arsenate and calcium ions showed little

TABLE V
Specificity of L-glutamate

Compound tested	Respired CO ₂ (per cent maximal activity)
None	5
L-Glutamate	100
L-Glutamine	88
D-Glutamate	11
γ -Aminobutyrate	19
L-Formylglutamate	22
DL- α -Methylglutamate	12
L-Aspartate	7
L-Asparagine	8
L-Alanine	11
L-Proline	14
L-Hydroxyproline	15
DL-Valine	8
L-Leucine	13
D-Glucosamine	4
L-Tyrosine	6
L-Arginine-HCl	8
DL-Methionine	9
DL-Threonine	15
L-Cysteine	27
L-Glutathione	20
DL-Serine	18

Each flask contained enzyme, DPN, thiamine pyrophosphate, MnCl_2 , phosphate buffer, pH 6.8, in the concentrations specified in Table III plus the amino compounds listed above at a concentration of 5 μ moles per flask. Incubations were carried out for 1 hour at 37°.

or no effect. Zinc and mercuric ions were highly toxic to the enzyme system. The inhibition by *p*-chloromercuribenzoate indicates that one or more of the enzymes in this system requires free sulfhydryl groups for activity. The action of hydroxylamine can most easily be explained by the removal of substrate.

Isolation and Determination of Intermediates—In crude homogenate preparations, the products of glyoxylate metabolism were identified as formate and CO_2 (Table I). The partially purified enzyme system produced only small amounts of formic acid as compared to the CO_2 formed. Excess formic acid, when added to the reaction mixture in trapping quantities, had no effect on CO_2 production; it also trapped negligible radioactivity as formic acid (Table VII). These data indicate that formate was not formed or destroyed at an appreciable rate by this enzyme preparation. If one postulates that a condensation between glyoxylate and L-glutamate occurred before decarboxylation, then a compound such as N-formylglutamic acid could be visualized as a product. An experiment was run with unlabeled formylglutamic acid as a trapping agent. The presence of formylglutamate had no influence on CO_2 evolution (Table VII). Paper chromatography of the deproteinized reaction mixture showed the presence of glutamic acid, formylglutamic acid, and a trace of glycine. To the remainder of the deproteinized reaction mixture was added 1.2 mmoles of carrier formylglutamate.

trichloroacetic acid used for deproteinization was removed by ether extraction and the ether removed with a stream of nitrogen. This solution was passed through a Dowex 50-X8 (acid form) column to remove amino acids. The eluate and washes from the Dowex 50 column were combined and neutral-

TABLE VI
Effect of inhibitors

Compound tested	Inhibitor added	Respired CO ₂ (per cent maximal activity)
None	μ moles	100
Na_2AsO_4	25	83
Versene	25	17
CaCl_2	8	100
ZnCl_2	8	7
HgCl_2	8	18
Hydroxylamine-HCl	12.5	3
NaCN	10	7
<i>p</i> -Chloromercuribenzoate	1	4
<i>p</i> -Chloromercuribenzoate + glutathione	1	3
DL- α -Methylglutamate	20	100

Each flask contained the complete system (Table III) plus the above compounds in the amounts shown. The flasks were incubated at 37° for 1 hour.

TABLE VII
Products of glyoxylate metabolism

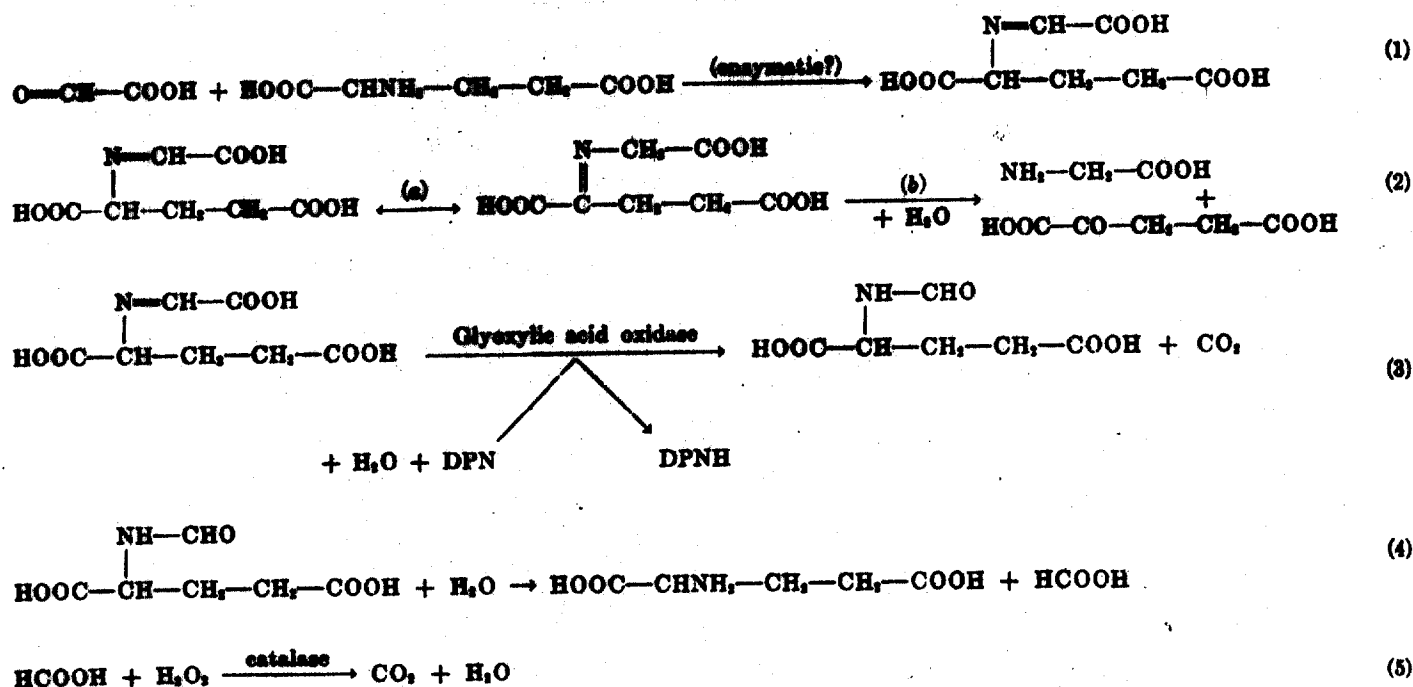
Substrates	Respired CO ₂	Formate	Formylglutamate
	μ moles	μ moles	μ moles
Glyoxylate-1,2- C^{14}	1.65		
Glyoxylate-1,2- C^{14} + formate (20 μ moles)	1.60	0.35	
Glyoxylate-1,2- C^{14} + formylglutamate (10 μ moles)	1.65		1.50

The usual complete system (Table III) with the addition of carrier formate or formylglutamic acid as indicated was used in these experiments. Incubations were carried out for 1 hour at 37°.

ized, and the acidic substances were adsorbed onto a column of Dowex 1-X10 (acetate form) and washed with several hundred ml. of water. 1.7 N acetic acid was passed through the column and 10 ml. fractions were collected (flow rate, 10 ml. per 20 minutes). Fractions containing formylglutamic acid were detected and identified by paper chromatography. The fractions containing formylglutamate were combined and evaporated to dryness *in vacuo*, taken up in ethanol, and again evaporated to dryness. The syrup was taken up in 2 ml. of ethanol and crystallized by the addition of 25 ml. of benzene. The isolated formylglutamate was hydrolyzed with H_2SO_4 for 1 hour and the formate was converted to CO_2 with mercuric sulfate, sulfuric acid reagent. The total radioactivity of this sample nearly equaled the radioactivity recovered in the respired CO_2 (Table VII). From these data it appears that the end products of this reaction were N-formylglutamic acid and CO_2 .

DISCUSSION

The experiments described show that a partially purified enzyme system isolated from rat liver mitochondria can catalyze the oxidative decarboxylation of glyoxylate. The details of the mechanism for this reaction are not yet clear. Thus, parts of the mechanism postulated below have as yet no basis in experi-



SCHEME 1

mental data, but known and circumstantial evidence would indicate that it must be close to the truth. The requirement for DPN, thiamine pyrophosphate, and manganous or magnesium ions, shows that part of the mechanism must be analogous to known oxidative decarboxylations. The almost absolute requirement for L-glutamic acid plus the isolation of N-formylglutamic acid as one of the decarboxylation products, adds a unique character to this system. Considering the specificity of L-glutamate for this reaction, there can be little doubt that this amino acid participates in this series of reactions at the enzyme level. L-Glutamate can be involved either before or after the decarboxylation step. If it entered this system after decarboxylation, then one would have to postulate that L-glutamate acts as a specific and necessary acceptor of the one carbon compound from the enzyme, in order to account for the nearly absolute requirement for L-glutamic acid. Although such a mechanism might be possible, the involvement of L-glutamate at a step before decarboxylation would seem more probable (Scheme 1). The first step is thought to be a rapid enzymatic condensation of glyoxylate and L-glutamate to form the hypothetical compound, N-glyoxylglutamic acid. The existence of such an intermediate is reasonable in view of the nonenzymatic transamination that has been shown to occur between these two compounds (15). Such a compound would be a Schiff's base with the possibility of electron shifts around the imino nitrogen to form the tautomers shown by Reaction 2a, Scheme 1. In the system under study, it is felt that the proposed intermediate, or its hydrated form, can be converted to CO_2 and N-formylglutamic acid by a DPN, thiamine-dependent enzyme that has been tentatively designated as glyoxylic acid dehydrogenase.

N-Formylglutamic acid has been shown to be one of the intermediates in the degradative pathway of histidine (10). It can also be synthesized by the reversible transformylation between the formylated form of citrovorum factor and L-glutamic acid

(18). The latter reaction, coupled with the formation of N-formylglutamic acid from glyoxylate, makes it possible to trace a logical pathway for the α -carbon of glycine to form the β -carbon of serine. This conversion can be envisioned as going from glycine \rightarrow glyoxylate \rightarrow N-formylglutamic acid \rightarrow formylated form of a folic acid derivative \rightarrow serine.

The enzymatic hydrolysis of N-formylglutamic acid (Reaction 4) was first observed by Tabor and Mehler (10), and subsequently reported by Ohmura and Hayaishi (19) and Kato *et al.* (20).

The oxidation of formate (Reaction 5) was shown to be catalyzed in mammalian tissues by the catalase-hydrogen peroxide complex (5).

The identification of N-glyoxylglutamic acid as an intermediate is under investigation, and a separation of the proposed enzyme activities is contemplated.

SUMMARY

1. Washed homogenates of rat liver can convert glyoxylate-1,2- C^{14} to formate and CO_2 . This oxidation was stimulated approximately 15-fold by the addition of L-glutamate.
2. The oxidation of glyoxylate was stimulated by L-glutamate in all tissues of the rat that were tested.
3. A partially purified preparation of enzyme that catalyzes the oxidative decarboxylation of glyoxylate has been obtained from rat liver mitochondria extracts. This system required diphosphopyridine nucleotide, thiamine pyrophosphate, L-glutamate and MnCl_2 to achieve maximal rates of decarboxylation. The optimal pH lies between pH 6.8 and 7. The maximal stimulation resulting from addition of L-glutamate occurred when the ratio of glyoxylate and L-glutamate concentration was one.
4. L-glutamate could not be replaced by any other amino acid. L-Glutamine was about one-half as effective, whereas little or no effect could be detected with other compounds, in-

cluding those structurally related to L-glutamate, such as D-glutamate, γ -aminobutyrate, DL- α -methylglutamate, L-aspartate or N-formylglutamate.

5. This enzyme system was inhibited by ethylenediamine-tetraacetate, zinc or mercuric ions, cyanide, hydroxylamine and *p*-chloromercuribenzoate, but not by calcium ions, α -methylglutamate, or N-formylglutamate.

6. The products of this oxidative decarboxylation were N-formylglutamic acid and CO₂.

7. Possible mechanisms of action are discussed.

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STUDIES ON THE VITAMIN B₁ ACTIVITY AND TOXICITY OF
THIAMINE MONONITRATE

The data presented in this report were collected to establish the relative vitamin B₁ activity and toxicity of thiamine mononitrate as compared with thiamine hydrochloride.

Vitamin B₁ Activity

In establishing the vitamin B₁ activity, two methods of assay were used. One was a rat curative assay, adapted from the procedure of M.I. Smith, in which weanling rats are maintained on a diet deficient in vitamin B₁ until typical "polyneuritis" occurred. At this point a single dose of the substance under test is administered and its effect on the "polyneuritis" is observed. In evaluating the results of this test, the percentage of animals cured together with the average duration of the cure and average weight gain in the animal are taken into consideration.

In the second method, the prophylactic method, weanling rats maintained on a vitamin B₁ free diet, are fed daily graded doses of the test substance or reference substance. An additional group of animals receiving only the basal diet serve as a control. The animals are maintained on this regimen for 8 weeks. The weight of each animal is recorded twice weekly and from these figures the average weight gain for each group is determined. By a comparison of the average weight gains in the test groups with those of reference groups, the relative vitamin B₁ activity of the test group is determined.

Table I is a summary of the results of the tests by each of the two methods. Complete protocols for the assays are attached.

From these data it is evident that thiamine mononitrate and thiamine hydrochloride possess a like order of vitamin B₁ activity.

Toxicity, acute

As an index of safety of thiamine mononitrate, its toxicity following single dose administration orally to mice and intravenously to mice and rats was determined and was compared with that of thiamine hydrochloride.

In determining the acute oral toxicity, the substances were made up in 50% gum acacia suspension and the required amounts of this suspension was administered by stomach tube to mice weighing from 20-25 grams each. To determine the intravenous toxicity the substances were dissolved in water in suitable concentration and administered to mice at a uniform rate of injection of 0.2 cc. per minute per 20 gram and to rats at a rate of 0.5 cc. per minute per 100 grams.

The results of these tests are given in table 2.

From the data in this table we estimate the L.D. 50 in mg. per kg. for these substances according to species and method of administration to be as follows:

	Mice		Rats
	<u>per os</u>	<u>intravenous</u>	<u>intravenous</u>
Thiamine mononitrate	7000	75	160
Thiamine hydrochloride	6000	85	140

It is apparent that the acute toxicities of thiamine mononitrate and thiamine hydrochloride are of the same order. Furthermore, when it is recalled that the above figures are in milligrams per kg. and that the daily requirements are in terms of micrograms per kg., it would appear that thiamine mononitrate, like thiamine hydrochloride, has a large factor of safety.

Table 1

<u>Curative Assay</u>	<u>Level fed (single dose) mcg.</u>	<u>No. of rats</u>	<u>No. of Cures</u>	<u>Av. duration of cure days</u>	<u>Av. wt. gain gm.</u>
Thiamine mononitrate	5+	20	12	8	9
Thiamine mononitrate	7+	11	8	9	8
Thiamine hydrochloride	5	25	17	9	9
Thiamine hydrochloride	7	20	17	11	11

<u>Prophylactic Assay</u>	<u>Level fed (daily)</u>	<u>No. of rats</u>	<u>Gain in Wt. 50 days gm.</u>
Controls (thiamine deficient)	-	10	No survivors
Thiamine mononitrate	5+	30	154
Thiamine mononitrate	10+	20	206
Thiamine hydrochloride	5	25	147
Thiamine hydrochloride	10	20	187

+ Equivalent of thiamine HCl

Table 2

<u>Substance</u>	<u>Species</u>	<u>Route of Administration</u>	<u>Dose mg./kg.</u>	<u>No. of animals</u>	<u>No. of deaths</u>
Thiamine Mononitrate	Mice	per os	4000	10	0
Thiamine Mononitrate	Mice	per os	6000	10	2
Thiamine Mononitrate	Mice	per os	8000	10	6
Thiamine Mononitrate	Mice	per os	10,000	10	8
Thiamine Mononitrate	Mice	per os	15,000	10	10
Thiamine Hydrochloride	Mice	per os	4000	10	0
Thiamine Hydrochloride	Mice	per os	6000	10	5
Thiamine Hydrochloride	Mice	per os	8000	10	9
Thiamine Hydrochloride	Mice	per os	10,000	10	10
Thiamine Mononitrate	Mice	intravenous	50	10	0
Thiamine Mononitrate	Mice	intravenous	75	10	5
Thiamine Mononitrate	Mice	intravenous	100	10	8
Thiamine Mononitrate	Mice	intravenous	150	10	10
Thiamine Hydrochloride	Mice	intravenous	50	10	0
Thiamine Hydrochloride	Mice	intravenous	75	10	2
Thiamine Hydrochloride	Mice	intravenous	100	10	9
Thiamine Hydrochloride	Mice	intravenous	150	10	10
Thiamine Mononitrate	Rats	intravenous	100	10	0
Thiamine Mononitrate	Rats	intravenous	150	10	4
Thiamine Mononitrate	Rats	intravenous	200	10	10
Thiamine Hydrochloride	Rats	intravenous	100	10	0
Thiamine Hydrochloride	Rats	intravenous	150	10	6
Thiamine Hydrochloride	Rats	intravenous	200	10	10

Prophylactic Assay - Thiamine Mononitrate Daily

<u>5 micrograms + daily</u>		<u>10 micrograms + daily</u>	
<u>Rat Nos.</u>	<u>Gain in wt. 56 days gm.</u>	<u>Rat Nos.</u>	<u>Gain in wt. 56 days gm.</u>
6	119	66	247
7	127	67	205
8	169	68	215
9	118	69	197
10	140	70	203
11	162	71	216
12	116	72	242
13	140	73	209
14	111	74	217
15	122	75	187
51	130	76-	177
52	155	77	222
53	130	78	182
54	141	79	187
55	150	80	163
56	163	111	183
57	224	112	237
58	151	113	186
59	156	114	182
60	195	115	170
61	204		
62	211		
63	175	Total	20
64	187		Av.
65	181		206.
106	160		
107	158		
108	125		
109	160		
110	166		
Total	Av.		
	154		
	71		

+ equivalent of thiamine HCl

NAS/NRC

COMPANY CODE

FPC No.
0222

Substance Reported *ANIMAL STUDIES 2*
Thiamine Mononitrate

X

THIAMINE MONONITRATE AND THIAMINE HYDROCHLORIDE

Thiamine mononitrate and thiamine hydrochloride have the same order of activity when bioassayed with rats for vitamin B₁ potency by the curative and prophylactic methods (Table I).

The acute oral toxicities in mice and in rats of thiamine mononitrate and of thiamine hydrochloride are the same. For all practical purposes the compounds may be considered as non-toxic, the LD 50 per os in mice is ca 45,000 times the daily requirement (Table II).

Chronic toxicity studies were carried out through 3 generations with rats of both sexes maintained from weaning to the age of six months on a stock ration (S₂) to which thiamine mononitrate or thiamine hydrochloride had been added at a level of 300 mg/kg of diet. This quantity of the vitamin represents a 300 to 400 fold increment over the rat's requirement for thiamine. The animals receiving either thiamine mononitrate or thiamine hydrochloride were indistinguishable both in growth and reproductive performance from their controls maintained on the natural food ration. Autopsy findings for all groups were the same (Table III).

Table I

Vitamin B₁ activity of thiamine mononitrate and thiamine hydrochloride

Curative Assay

	<u>Level fed</u> <u>(single dose)</u> micrograms	<u>No. of</u> <u>rats</u>	<u>No. of</u> <u>cures</u>	<u>Av.</u> <u>duration</u> <u>of cure</u> days	<u>Av. wt.</u> <u>gain</u> gm.
thiamine mononitrate	5*	20	12	8	9
thiamine mononitrate	7*	11	8	9	8
thiamine hydrochloride	5	25	17	9	9
thiamine hydrochloride	7	20	17	11	11

Prophylactic assay

	<u>Level fed</u> <u>(daily)</u> micrograms	<u>No. of</u> <u>rats</u>	<u>Gain in wt.</u> <u>56 days</u> gm.
controls (thiamine deficient)	-	10	No survivors
thiamine mononitrate	5*	30	154
thiamine mononitrate	10*	20	206
thiamine hydrochloride	5	25	147
thiamine hydrochloride	10	20	187

* equivalent of thiamine hydrochloride

Table II

Acute toxicity of thiamine mononitrate and
thiamine hydrochloride (experiments
carried out by Mr. S. Kuna)

LD50 mgm/kgm

Mice

(groups of 10 each)

I.V.

per os

Rats

(groups of 5 each)

I.V.

thiamine mononitrate

75

7,000

140

thiamine hydrochloride

85

6,000

140

Table III

Chronic toxicity studies with thiamine mononitrate
and thiamine hydrochloride

GROWTH

Diet	No. of rats and sex*	Av. gain in weight 180 days gm.	Av. daily intake of vitamin at 180 days mg.
<u>S₂ A</u> 300 mg thiamine mononitrate/kg diet			
1st generation	25 males 24 females	365 198	- -
2nd generation	33 males 22 females	348 214	6.3 4.8
3rd generation	36 males 30 females	385 213	7.2 5.4
<u>S₂ B</u> 300 mg thiamine hydrochloride/kg diet			
1st generation	25 males 25 females	316 210	- -
<u>Diet Ration</u>			
<u>S₂ B</u>			
2nd generation	30 males 10 females	345 204	6.3 5.4
3rd generation	45 males 45 females	377 204	7.5 5.1
<u>S₂</u>	No. of rats and sex	Av. gain in wt. 180 days gm.	
1st generation	15 males 5 females	350 202	
2nd generation	35 males 30 females	380 205	

	<u>No. of rats and sex</u>	<u>Av. gain in wt. 180 days</u> gm.
3rd generation	50 males 50 females	381 205

* the females employed in the growth studies were not bred.

Reproduction (females)

<u>Ration</u>	<u>No. of female rats</u>	<u>No. bred</u>	<u>No. implanted</u>	<u>No. litters</u>	<u>Av. no. young/ litter</u>	<u>Av. wt. of young at birth</u> gm.	<u>Av. wt. at weaning</u> gm.
S ₂ A							
1st generation	25	23	19	17	9	5.6	32
2nd generation	46	44	43	41	8	5.3	33
S ₂ B							
1st generation	13	12	10	10	10	5.6	34
2nd generation	26	25	25	24	10	5.6	35
S ₂							
1st generation	14	12	11	10	12	5.8	27
2nd generation	35	35	34	29	10	5.9	34

* mothers were allowed to suckle 3 young

Reproduction (males)

<u>Ration</u>	<u>No. of rats</u>	<u>No. of positive matings*</u>
S ₂ A		
1st generation	25	25
2nd generation	43	43
S ₂ B		
1st generation	10	9
2nd generation	34	32
S ₂		
1st generation	10	10
2nd generation	32	31

* males were placed with gestruc females maintained on diet S₂

Prophylactic Assay - Thiamine Hydrochloride Daily

<u>5 micrograms daily</u>		<u>10 micrograms daily</u>	
<u>Gain in wt.</u>		<u>Gain in wt.</u>	
<u>Rat Nos.</u>	<u>56 days</u>	<u>Rat Nos.</u>	<u>56 days</u>
	gm.		gm.
11	134	26	214
12	145	27	153
13	137	28	177
14	195	29	206
15	151	30	190
16	134	31	211
17	212	32	156
18	129	33	159
19	134	34	207
20	135	35	193
21	158	36	192
22	165	37	195
23	155	38	207
24	153	39	196
25	167	40	184
31	133	96	198
32	146	97	183
33	136	98	170
34	133	99	177
35	164	100	164
36	134		
37	123		
38	131		
39	96		
40	182		
—	—		
Total 25	Av. 147	Total 20	Av. 187

Curative Assay

5 Micrograms* Thiamine mononitrate (single dose)

<u>Rat Nos.</u>	<u>Response**</u>	<u>Gain in Wt. gm.</u>	<u>Duration of cure days</u>
176	C	15	12
332	NC	--	--
335	C	6	6
337	C	9	7
360	C	15	6
361	C	10	10
367	NC	--	--
373	NC	--	--
374	C	8	6
377	NC	--	--
380	C	7	10
490	C	4	5
494	C	2	4
561	NC	--	--
563	NC	--	--
593	C	8	6
596	C	4	6
600	C	12	9
608	NC	--	--
653	NC	--	--
<hr/>		<hr/>	<hr/>
Total 20		Av. 9	Av. 8.0
12 C			
8 NC			

* Equivalent of thiamine HCl

** C - indicate cure

NC - indicate no cure

Curative Assay

5 Micrograms Thiamine hydrochloride (single dose)

<u>Rat Nos.</u>	<u>Response**</u>	<u>Gain in Wt. gm.</u>	<u>Duration of cure days</u>
244	C	6	14
267	C	28	27
322	NC	--	--
333	NC	--	--
339	C	14	5
364	C	10	9
365	NC	--	--
366	C	11	5
367	NC	--	--
368	C	6	6
370	C	8	10
372	C	11	7
374	C	5	11
377	C	8	9
380	C	8	7
394	C	7	7
523	C	8	8
566	NC	--	--
592	C	9	7
571	NC	--	--
583	C	7	5
606	C	8	7
622	NC	--	--
623	C	5	6
629	NC	--	--
<hr/>		<hr/>	<hr/>
Total 25		Av. 9	Av. 9
17 C			
8 NC			

** C - cure
NC - no cure

Curative Assay

5 Micrograms Thiamine hydrochloride (single dose)

<u>Rat Nos.</u>	<u>Response**</u>	<u>Gain in Wt. gm.</u>	<u>Duration of cure days</u>
244	C	6	14
267	C	28	27
322	NC	--	--
333	NC	--	--
339	C	14	5
364	C	10	9
365	NC	--	--
366	C	11	5
367	NC	--	--
368	C	6	6
370	C	8	10
372	C	11	7
374	C	5	11
377	C	8	9
380	C	8	7
394	C	7	7
523	C	8	8
556	NC	--	--
562	C	9	7
571	NC	--	--
583	C	7	5
606	C	8	7
622	NC	--	--
623	C	5	6
629	NC	--	--
<hr/> Total 25 17 C 8 NC		<hr/> Av. 9	<hr/> Av. 9

** C - cure
 NC - no cure

Curative Assay

7 Micrograms* Thiamine mononitrate (single dose)

<u>Rat Nos.</u>	<u>Response**</u>	<u>Gain in Wt. gm.</u>	<u>Duration of cure days</u>
322	C	9	10
362	C	3	9
364	C	10	9
367	C	10	4
370	C	2	7
372	C	14	12
374	C	12	13
494	NC	--	--
539	NC	--	--
554	NC	--	--
556	C	2	8
<hr/> Total 11		<hr/>	<hr/>
8 C			
3 NC			
		Av. gain 9	Av. duration 9

* equivalent of thiamine HCl

** C - indicates cure

NC - indicates no cure

Curative Assay

7 Micrograms* Thiamine hydrochloride (single dose)

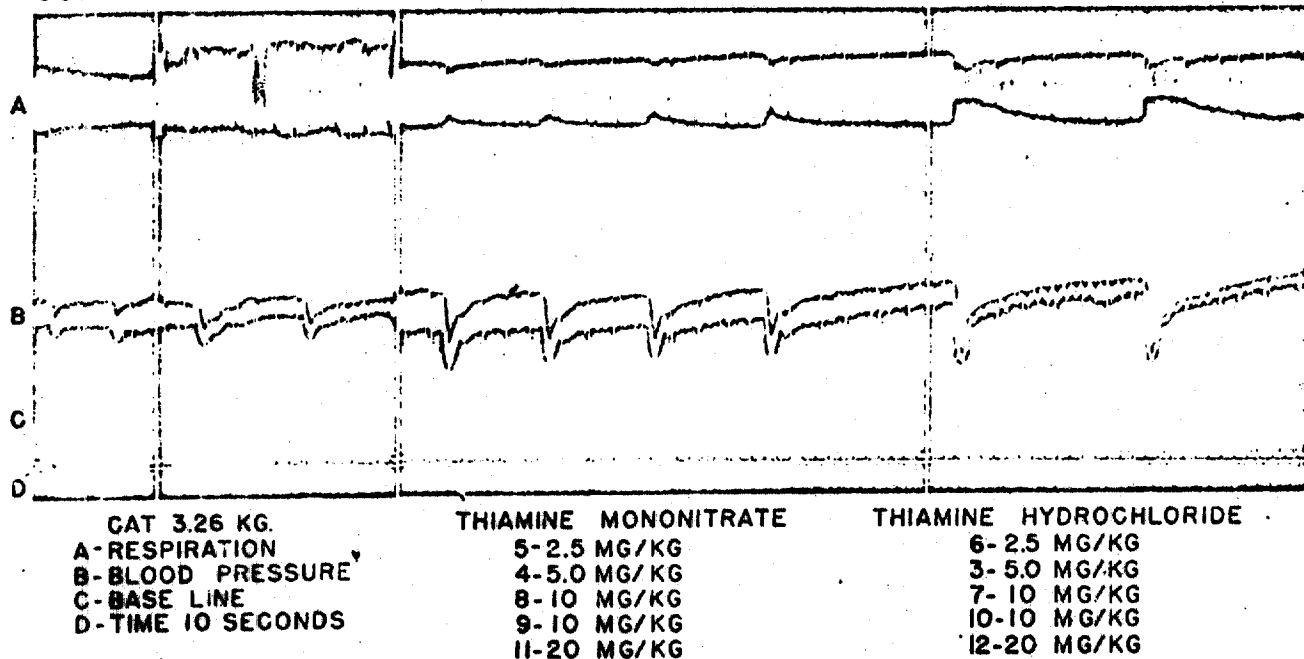
<u>Rat Nos.</u>	<u>Response**</u>	<u>Gain in Wt. gm.</u>	<u>Duration of cure days</u>
256	C	7	9
257	C	13	9
322	C	9	9
326	NC	--	--
332	C	26	31
337	C	16	11
338	C	17	8
357	C	11	10
360	C	10	9
361	C	9	11
363	C	13	10
366	C	7	13
367	C	13	11
370	C	11	12
372	C	8	13
377	C	8	11
380	C	8	10
490	C	6	5
494	NC	--	--
525	NC	--	--
<hr/> Total 20 17 C 3 NC		Av. 11	Av. 11

* equivalent of thiamine HCl

** C - cure

NC - no cure

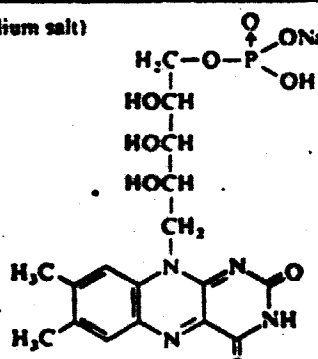
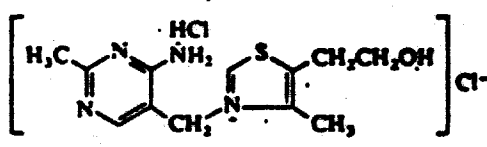
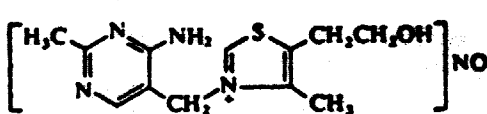
COMPARISON OF THIAMINE MONONITRATE TO THIAMINE HYDROCHLORIDE



From the above tracing it is evident that there is no difference between thiamine hydrochloride and thiamine mononitrate in their effect on blood pressure and respiration.

Vitamins—(Continued)

Table 1. Properties—(Continued)

Compound	Formula	Properties	Solubility (g 100 ml)	Stability
RIBOFLAVIN PHOSPHATE (sodium salt) Vitamin B ₂ phosphate sodium Riboflavin 5-phosphate sodium Flavin mononucleotide Riboflavin 5-phosphate ester monosodium salt	 $C_{17}H_{20}N_4O_6PNa \cdot 2H_2O$ mol. wt. 514.37	Orange-yellow crystals: $[\alpha]_D^{20} = +38$ to 42° (20°, HCl).	4-11, water (depending on pH).	Similar to riboflavin.
THIAMINE Thiamine chloride hydrochloride Vitamin B hydrochloride Aneurine (hydrochloride) Cryzamin Anti-beriberi vitamin 3-(4-Amino-2-methylpyrimidyl-5-methyl)-4-methyl-5-(β-hydroxyethyl) thiazolium chloride hydrochloride	 $C_{12}H_{17}ClN_4OS \cdot HCl$ mol. wt. 337.28	White monoclinic crystals: m.p. 246-250°C (decomposes): $\lambda_{max} = 246 m\mu$ (0.1 N HCl): $E_{1\%}^{1cm} = 410$: optically inactive: 1 mg = 333 IU.	100, water: 1, alcohol: ins. org. sol.	Stable when dry, stable in acid, una. at alkaline pH: to prolonged heating, presence of bisulfite or thiaminase: very hygroscopic.
Thiamine Mononitrate Aneurine mononitrate Vitamin B. mononitrate	 $C_{12}H_{17}N_4O_6S$ mol. wt. 327.36	White crystals: m.p. 196-200°C (decomposes): less hygroscopic than chloride hydrochloride. 1 mg = 343 IU.	2.7 water: ins. org. solv.	More stable than chloride salt in dry products: not hygroscopic.

Handbook of Biochemistry

Vitamins—(Continued)

Table 2. Biological Characteristics—(Continued)

Compound	Function	Deficiency symptoms	Hyper-use symptoms	Coenzyme and enzyme involved	Remarks
Thiamine	Functions as a coenzyme: activation and transfer of active acetaldehyde, glyceraldehyde and succinic semialdehyde; functions in carbohydrate metabolism	Polyneuritis, beriberi, convulsions, muscle paralysis, anorexia, bradycardia, heart dilation, myocardial lesions, retarded growth, edema, pyruvic acid accumulation in blood and tissues	Analgesic effect on peripheral nerves, vascular hypertension	Cocarbonylase, transketolase, carboxylases	Like most water-soluble vitamins, there is no significant tissue storage. Amprolium, pyri-thiamine, oxythiamine and others are antimetabolites. The two important forms in production are the hydrochloride and the mononitrate.

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Vitamins—(Continued)

TABLE 3. ANIMAL REQUIREMENTS (per kilo ration) (NAS-NRC)

Animal	Purpose	A (IU)	D (IU)	E (IU)	K (mg)	AA (mg)	Bi (μg)	Ch (mg)	Cy (μg)	FA (mg)	Ni (mg)	Pa (mg)	Py (mg)	Ri (mg)	Th (mg)
Cat (1962)	Growing	25,000	1,000	34-136	-	-	-	3,000	-	-	40	5	2	4	4
Dog (1952, revised)	Growing	5,000	250	50 ^a	-	-	-	1,200	22	0.18	10	2.2	1	2.2	0.7
Fox ^a (1953)	Growing	2,410	-	-	-	-	-	-	-	0.2	10	8	2	2.6	1.0
Guinea pig (1962)	Growing	16,000	-	60	10	200	-	1,500	-	-	50	20	4	16	6
Hamster (1962)	Growing	13,000	-	25	-	-	-	-	-	-	-	40	6	6	6
Mink ^a (1968)	Growing	3,500	-	25	-	-	-	-	30	0.5	20	6	1.1	1.5	1.2
Monkey	Growing	-	-	-	-	25	250	-	25	1.3	38	-	1.2	0.8	0.8
Mouse (1962)	Growing	500	150	20	-	-	-	570-1,140	5	-	30	8.5	1	4	2.9
	Pregnancy and lactation	500	-	-	-	-	-	-	4.5	-	-	10.2	-	7	5
Rat (1962)	Growing	2,000	-	60	1	-	-	750	5	-	15	8	1.2	2.5	1.3
	Gestation	12,000	-	30	-	-	-	<1,000	5	-	-	8	0.6	4	4
	Lactation	12,000	-	20	-	-	-	<1,000	5	-	-	10	0.6	4	4
Poultry															
Chicken (1966)	Growing 0-8 weeks	2,000	200	-	0.5	-	90	1,300	9	1.2	27	10	3	3.6	1.8
	Growing 8-16 weeks	2,000	200	-	-	-	-	-	-	-	11	10	-	1.8	-
	Laying	4,000	500	-	-	-	-	-	-	0.25	-	2.2	-	2.2	-
	Breeding	4,000	500	-	-	-	150	-	3	0.35	-	10	4.5	3.8	0.8
Duck (1966)	Growing	-	220	-	-	-	-	-	-	-	55	11	2.6	4	-
Pheasant (1966)	Growing	-	1,200	-	-	-	-	-	-	-	60	-	-	3.5	-
Quail (1966)	Growing	13,000	-	-	-	-	-	-	-	-	-	-	-	-	-
Turkey (1965)	Growing 0-8 weeks	4,000	900	-	0.7	-	-	1,900	3	0.9	70	11	3	3.6	-
	Growing 8-16 weeks	4,000	900	-	-	-	-	-	-	-	-	-	-	-	-
	Breeding	4,000	900	-	-	-	-	-	-	0.8	-	16	-	3.8	-
Swine (1968)	Growing 5-10 ^a	2,200	220	-	-	-	-	1,000	22	0.5-1	22	13	1.5	3	1.3
	Growing 10-20	1,750	200	-	-	-	-	900	15	0.5-1	18	11	1.5	3	1.1
	Growing 20-35	1,300	200	-	-	-	-	-	11	-	14	11	1.1	2.6	1.1
	Growing 35-60	1,300	125	-	-	-	-	-	11	-	10	11	-	2.2	1.1
	Growing 60-100	1,300	125	-	-	-	-	-	11	-	10	11	-	2.2	1.1
	Breeding 110-160	4,100	275	-	-	-	-	-	13.8	-	22	16.5	-	4.4	1.4
	Lactating 140-200	3,300	220	-	-	-	-	-	11	-	17.6	13.2	-	3.3	1.1
	Boars 110-150	4,100	275	-	-	-	-	-	13.8	-	22	16.5	-	4.1	1.4

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^a AA, Ascorbic acid; Bi, Biotin; Ch, Choline; Cy, Cyanocobalamin; FA, Folic acid; Ni, Niacin; Pa, Pantothenic acid; Py, Pyridoxine; Ri, Riboflavin; Th, Thiamine.

^b Required but specific needs not established. AA only a dietary essential for monkey and guinea pig.

^c NAS-NRC publications also exist for the vitamin requirements of large animals.

^d Kilograms

^e Not a dietary essential.

^f From 7-31 weeks of age, male body wt. goes from 690 to 2,070 g with consumption of 27 to 75 g of dry food daily;

female body wt. goes from 560 to 1,110 g with consumption of 32 to 60 g of dry food daily.

^g From 7-31 weeks of age, male body wt. goes from 1.4 to 5.7 kg with consumption of 39 to 160 g of dry food daily; female body wt. goes from 1.3 to 4.7 kg with consumption of 36 to 118 g of dry food daily.

^h Growing.

Vitamins—(Continued)

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RECOMMENDED DIETARY ALLOWANCES
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THIAMIN

Thiamin functions in carbohydrate metabolism as a coenzyme in the decarboxylation of α -keto acids and in the utilization of pentose in the hexose monophosphate shunt. Pyruvic acid tends to accumulate in tissues in severe thiamin deficiency, and reduced transketolase activity in the red blood cells has been observed in subjects consuming diets of poor thiamin content.^{1,2}

It has generally been assumed that thiamin need is related to calorie need, particularly to those calories derived from carbohydrate. Although dietary fat does "spare" thiamin to some extent,³ the reduction in requirement appears to be small.⁴ Older estimates of minimal thiamin requirement approximated 0.2 mg/1,000 kcal.⁵⁻¹² A joint FAO/WHO Expert Group¹³ reviewed newer evidence together with epidemiological studies and concluded that 0.35 mg of thiamin per 1,000 kcal represents the requirement, and they recommended 0.4 mg/1,000 kcal to take care of individual variation. The same requirement was estimated from measurement of urinary excretion of thiamin metabolites.¹⁴ It is considered that 0.5 mg/1,000 kcal will maintain satisfactory thiamin nutriture under normal conditions in the United States, and this forms the base of the current recommendations.

The nature of the relationship that exists between thiamin excretion and thiamin intake per 1,000 kcal is still debatable.^{5,15,16} Because it is possible that older persons use thiamin less efficiently, it is deemed advisable to recommend that a thiamin intake of 1.0 mg/day be maintained by older adults, even though they are consuming less than 2,000 kcal daily.

The literature on thiamin needs in maternal and child nutrition suggests an increased need for thiamin during pregnancy.^{17,18} Although the magnitude of the increase is uncertain, an additional allowance of 0.2 mg/day is recommended during pregnancy, in accordance with the increased calorie recommendation.

The thiamin content of human milk is variable and is influenced by thiamin intake.¹⁹⁻²¹ Mature human milk provides an average of 0.015 mg of thiamin/100 ml, as compared with 0.04 mg/100 ml in cows' milk. Assuming that the lactating woman secretes 850 ml/day, the total thiamin output would be around 0.13 mg. It is considered

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that the allowance of 0.5 mg/1,000 kcal also applies to the lactating woman, and the recommended thiamin allowance is set accordingly.

The minimum thiamin requirement for the human infant,²²⁻²⁵ based on intake from mother's milk, cows' milk formulation, and urinary-excretion studies, appears to be approximately 0.2 mg/1,000 kcal. The RDA is 0.5 mg/1,000 kcal. The recommended allowances for children have been calculated as 0.5 mg/1,000 kcal. This level of intake maintains whole-blood thiamin levels and permits relatively high urinary thiamin excretions.^{26,27}

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RECOMMENDED DAILY DIETARY ALLOWANCES,* Revised 1968

Designed for the maintenance of good nutrition of practically all healthy people in the U.S.A.

AGE ^b (years)	WEIGHT		HEIGHT		kcal	PROTEIN (gm)	FAT-SOLUBLE VITAMINS		WATER-SOLUBLE VITAMINS					MINERALS										
							VITA- MIN A	VITA- MIN D	VITA- MIN E	ASCOR- BIC	FOLA- CIN ^c	NIA- CIN	RIBO- FLAVIN	THIA- MIN	VITA- MIN B ₆	VITA- MIN B ₁₂	CAL- CIUM	PHOS- PHORUS	IODINE	IRON	MAG- NESIUM			
	ACTIVITY (IU)	(IU)	(IU)	ACID (mg)			(mg)	(mg equiv) ^d	(mg)	(mg)	(μg)	(g)	(g)	(μg)	(mg)	(mg)								
From	Up to	(kg)	(lbs)	cm	(in.)																			
Infants	0-1/6	4	9	55	22	kg X 120	kg X 2.2 ^e	1,500	400	5	35	0.05	5	0.4	0.2	0.2	1.0	0.4	0.2	25	6	40		
	1/6-1/2	7	15	65	25	kg X 110	kg X 2.0 ^e	1,500	400	5	35	0.05	7	0.5	0.4	0.3	1.5	0.5	0.4	40	10	60		
	1/2-1	9	20	72	28	kg X 100	kg X 1.8 ^e	1,300	400	5	35	0.1	8	0.6	0.5	0.4	2.0	0.6	0.5	45	15	70		
Children	1-2	12	26	81	32	1,100	25	2,000	400	10	40	0.1	8	0.6	0.6	0.5	2.0	0.7	0.7	55	15	100		
	2-3	14	31	91	36	1,250	25	2,000	400	10	40	0.2	8	0.7	0.6	0.6	2.5	0.8	0.8	60	15	150		
	3-4	16	35	100	39	1,400	30	2,500	400	10	40	0.2	9	0.8	0.7	0.7	3	0.8	0.8	70	10	200		
	4-6	19	42	110	43	1,600	30	2,500	400	10	40	0.2	11	0.9	0.8	0.9	4	0.8	0.8	80	10	200		
	6-8	23	51	121	48	2,000	35	3,500	400	15	40	0.2	13	1.1	1.0	1.0	4	0.9	0.9	100	10	250		
	8-10	24	62	131	52	2,200	40	3,500	400	15	40	0.3	15	1.2	1.1	1.2	5	1.0	1.0	110	10	250		
Males	10-12	35	77	140	55	2,500	45	4,500	400	20	40	0.4	17	1.3	1.3	1.4	5	1.2	1.2	125	10	300		
	12-14	45	95	151	59	2,700	50	5,000	400	20	45	0.4	18	1.4	1.4	1.6	5	1.4	1.4	135	18	350		
	14-18	59	130	170	67	3,000	60	5,000	400	25	55	0.4	20	1.5	1.5	1.8	5	1.4	1.4	150	18	400		
	18-22	67	147	175	69	2,400	60	5,000	400	30	60	0.4	18	1.6	1.4	2.0	5	0.8	0.8	140	10	400		
	22-35	70	154	175	69	2,800	65	5,000	—	30	60	0.4	18	1.7	1.4	2.0	5	0.8	0.8	140	10	350		
	35-55	70	154	173	68	2,600	65	5,000	—	30	60	0.4	17	1.7	1.3	2.0	5	0.8	0.8	125	10	350		
	55-75+	70	154	171	67	2,400	65	5,000	—	30	60	0.4	14	1.7	1.2	2.0	6	0.8	0.8	110	10	350		
Females	10-12	35	77	142	56	2,250	50	4,500	400	20	40	0.4	15	1.3	1.1	1.4	5	1.2	1.2	110	18	300		
	12-14	44	97	154	61	2,300	50	5,000	400	20	45	0.4	15	1.4	1.2	1.6	5	1.3	1.3	115	18	350		
	14-16	52	114	157	62	2,400	55	5,000	400	25	50	0.4	16	1.4	1.2	1.8	5	1.3	1.3	120	18	350		
	16-18	54	119	160	63	2,300	55	5,000	400	25	50	0.4	15	1.5	1.2	2.0	5	1.3	1.3	115	18	350		
	18-22	58	128	163	61	2,000	55	5,000	400	25	55	0.4	13	1.5	1.0	2.0	5	0.8	0.8	100	18	350		
	22-35	58	128	163	64	2,000	55	5,000	—	25	55	0.4	13	1.5	1.0	2.0	5	0.8	0.8	100	18	300		
	35-55	58	128	160	63	1,850	55	5,000	—	25	55	0.4	13	1.5	1.0	2.0	5	0.8	0.8	90	18	300		
	55-75+	58	128	157	62	1,700	55	5,000	—	25	55	0.4	13	1.5	1.0	2.0	6	0.8	0.8	80	10	300		
Pregnancy						+200	65	6,000	400	30	60	0.8	15	1.8	+0.1	2.5	8	+0.4	+0.4	125	18	450		
Lactation						+1,000	75	8,000	400	30	60	0.5	20	2.0	+0.5	2.5	6	+0.5	+0.5	150	18	450		

* The allowance levels are intended to cover individual variations among most normal persons as they live in the United States under usual environmental stresses. The recommended allowances can be attained with a variety of common foods, providing other nutrients for which human requirements have been less well defined. See text for more-detailed discussion of allowances and of nutrients not tabulated.

^b Entries on lines for age range 22-35 years represent the reference man and woman at age 22. All other entries represent allowances for the midpoint of the specified age range.

* The folacin allowances refer to dietary sources as determined by *Lactobacillus casei* assay. Pure forms of folacin may be effective in doses less than 1/4 of the RDA.

^c Niacin equivalents include dietary sources of the vitamin itself plus 1 mg equivalent for each 60 mg of dietary tryptophan.

^d Assumes protein equivalent to human milk. For proteins not 100 percent utilized factors should be increased proportionately.

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Studies of Thiamine Metabolism in the Rat¹

1. METABOLIC PRODUCTS FOUND IN URINE

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ABSTRACT The catabolic products of the metabolism of pyrimidine carbon C¹⁴-labeled thiamine in rat and rabbit urine were examined by the application of column and paper chromatographic techniques. The presence of a minimum of 22 different metabolites of thiamine was detected. It is considered that most of these are "true" metabolites since at the pH of rat urine and at room temperature, thiamine was quite stable.

There is abundant literature concerning the question of thiamine balance in both experimental animals and man, but little is known of the metabolic fate of the thiamine molecule. A variety of forms of thiamine (other than thiamine phosphate and "active aldehydes") have been shown with reasonable certainty to be present in the tissues and excretory products of mammals. These include free thiamine, thiochrome, thiamine disulfide (1), 5-(2-hydroxyethyl)-4-methylthiazole (1-3), and of necessity, some form of the pyrimidine moiety of thiamine. Early reports suggesting that thiamine pyrophosphate is present in mammalian urine (4, 5)² have not been confirmed by more recent investigators (1, 6, 7). A claim for the presence of thiamine 5-acetic acid in human urine (8) has recently been withdrawn (6). The isolation from a perfusate of rat liver of thiamine disulfate, o-acetyl thiamine, and a second acetyl derivative in which the acetyl group was not bound to oxygen (9, 10) has recently been reported. At least a dozen unidentified degradation products of C¹⁴ thiazole-labeled thiamine have been reported by Iacono et al.³ and Iacono and Johnson (1) to occur in rat urine.

Although several groups of workers (11-13) have provided indirect evidence for the occurrence of some form of the pyrimidine moiety of thiamine in urine, only Kawasaki and Okada (14) have succeeded in isolating such a compound. These workers reported the isolation of 2-methylamino-5-hydroxymethyl pyrimidine from

human urine after giving large doses of thiamine to test subjects. Whether the isolated compound was really a metabolic product of thiamine metabolism appears to be open to some doubt. The authors themselves point out that the extremes of temperature and pH used during their isolation procedure may well have led to the generation of their compound by hydrolytic cleavage of thiamine present in the urine. In addition, Suhara and Iritani (15) have recently reported they were unable to detect the presence of this compound in the urine of rats.

The present study was undertaken particularly to clarify the metabolism of the pyrimidine moiety of thiamine. This was accomplished by the use of thiamine labeled with C¹⁴ in the pyrimidine part of the molecule.

EXPERIMENTAL PROCEDURE

Except where noted, adult female Sprague-Dawley rats were used. They were housed in pairs in stainless steel metabolism cages constructed to permit the separate collection of urine and feces. Twenty-four-hour urine specimens were collected under toluene in glass bottles

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² Iacono, J. M., G. Wolf and B. C. Johnson 1953 Metabolism of radioactive thiamine in the rat. *Federation Proc.*, 12: 223 (abstract).

containing 1 ml of 2 N acetic acid and a few crystals of thymol. The samples of urine were then pooled, centrifuged to remove any precipitate, and stored at -20° until ready for use.

A thiamine-deficient basal diet was used throughout the experiment. It had the following percentage composition: vitamin-free casein, 18; cottonseed oil, 5; sucrose, 69; succinylsulfathiazole,³ 1; non-nutritive fiber,⁴ 3; and salt mixture,⁵ 4. The following vitamins were added at the levels indicated: (mg/100 g of basal diet) vitamins A and D, at 400 and 40, I.U. respectively; riboflavin, 0.80; niacin, 4.00; Ca pantothenate, 4.00; biotin, 0.04; folic acid, 0.20; pyridoxine, 0.50; p-aminobenzoic acid, 10.00; choline chloride, 100.00; α -tocopheryl acetate 4.0; and menadione, 0.5.

A single New Zealand-strain rabbit was used in one short experiment. This animal was housed in a standard metabolic cage and fed a stock pelleted diet. Urine was collected for 12-hour periods, under toluene, in a glass bottle containing 5 ml of 2 N acetic acid and a few crystals of thymol.

In all experiments the animals were allowed food and water ad libitum.

The synthesis of thiamine labeled with C^{14} in the pyrimidine ring was accomplished using the pyrimidine synthesis described by Grewe (16). The method used for coupling the pyrimidine and thiazole moieties was that of Gravin (17). Since malonitrile-1- C^{14} was used in this synthesis, 2 species of labeled thiamine molecules were produced. One species was labeled in the 4-position of the pyrimidine ring and the other was labeled in the methylene bridge. The specific activity of the thiamine- C^{14} synthesized was 0.296 μ c/ μ mole. The final product showed an ultraviolet spectrum similar to that of authentic thiamine chloride hydrochloride at 2 pH values. The molecular extinction coefficients of both compounds were the same and a mixed melting point determination showed no depression. Both the authentic and labeled compounds behaved identically on ion exchange and paper chromatography. The labeled compound yielded a fluorescent product on alkaline oxidation that migrated on paper chro-

matography with the same R_f value as authentic thiochrome. Finally, the synthesized compound supported the growth of *Lactobacillus viridescens* and *Phycomyces blakesleeanus*, cured acute thiamine deficiency in the rat, and supported the normal growth of rats maintained with a thiamine deficient diet for 10 months.

Acid-washed charcoal was used to obtain the urinary metabolites of thiamine, C^{14} in a form free of inorganic salts. The charcoal⁶ was prepared by refluxing 100 g of charcoal for 3 hours in one liter of 2 N hydrochloric acid. The charcoal was allowed to settle, the hydrochloric acid decanted, and the same procedure was repeated. After recovery of the charcoal by Büchner filtration the charcoal was washed exhaustively with distilled water until the pH of the washings was 5.0 or greater. It was then washed with 4 liters of ethanolic ammonia (ethanol/ammonium hydroxide/water, 50/5/45, by volume) and with distilled water until neutral before air-drying. In the desalting procedure the pH of the urine was adjusted to 6.0 to 6.5, 1 g of acid-washed charcoal added for every 100 ml of urine, and the suspension stirred for 1 hour at room temperature. The charcoal was removed by centrifugation ($5000 \times g$ for 10 minutes) and suspended in 100 ml of pyridine/ethanol/water (10/45/45 by volume) for each gram of charcoal originally added to the urine. This mixture was shaken for 3 hours at 37° , centrifuged, and the supernatant containing the radioactive metabolites was decanted. Some 85 to 90% of the radioactivity in the urine could be obtained free of inorganic salts by this procedure.

For chromatography the charcoal eluates were reduced in volume in a rotary evaporator under vacuum at 40° to approximately 5 ml. The flask was washed with 5 ml of distilled water which was added to the original concentrate and the pH was adjusted to 5.5. After centrifugation at $5000 \times g$ for 10 minutes the super-

³ Nutritional Biochemicals Corporation, Cleveland.

⁴ Alphacel, Nutritional Biochemicals Corporation, Cleveland.

⁵ Hubbel, R. B., L. B. Mendel and A. J. Wakeman. 1937. A new salt mixture for use in experimental diets. *J. Nutrition*, 14: 273.

⁶ Norite-A, Matheson Coleman and Bell, East Rutherford, New Jersey.

nantant was placed on an Amberlite CG-50 column⁷ (1.0 × 40.0 cm; 200–400 mesh; H⁺ form) which had been previously washed with distilled water until the pH of the eluant was 4.5 or greater. After all of the sample had drained into the resin two 2-ml portions of distilled water were permitted to drain into the resin and the radioactive metabolites were eluted with water to 0.01 N hydrochloric acid gradient. Five hundred milliliters of eluant were used and 10- to 12-ml fractions collected.

To prepare the radioactive peaks from the Amberlite CG-50 columns for paper chromatography, the combined column fractions were reduced in volume under vacuum at 40° to approximately 3 ml and then lyophilized. The lyophilizate was dissolved in 0.1 ml of the chromatographic solvent system to be used and applied to the paper in 3-cm bands at a point 4 cm from the bottom of the filter paper sheet.

It was found necessary to subject the initial radioactive peak from the Amberlite CG-50 columns to further ion exchange chromatography on a neutral column of Dowex-1-resin⁸ (1.0 × 20.0 cm; 100–200 mesh; chloride form) prior to paper chromatography. This was accomplished by reducing the combined fractions of the peak to approximately 5 ml under vacuum at 40°, adjusting to pH 6.5, and placing on the Dowex-1 column. After the sample had passed into the column the resin was washed twice with 2-ml portions of water and the column eluted first with 100 ml of distilled water followed by 0.001 N hydrochloric acid.

Ascending paper chromatography was performed on acid-washed sheets of Whatman filter paper (nos. 1 and 3 MM). The sheets were acid-washed by descending chromatography with 500 ml of 0.1 N hydrochloric acid followed by 500 ml of 50% (by volume) ethanol.

The metabolites of thiamine-C¹⁴ were detected by radioautography, by fluorescence or quenching under ultraviolet light, and by a combination of these methods.

Radioautographs were prepared using Eastman "No-screen" X-ray film. The film was exposed to the paper chromatogram for periods varying from 2 to 30 days

depending on the amount of radioactivity and developed in the conventional manner.

Radioactivity measurements were made using a Nuclear-Chicago D-47 gas-flow detector equipped with a model M5 sample changer, or a Packard Tri-Carb liquid scintillation counter.

The fractions collected from the ion exchange columns were checked for radioactivity by plating 0.5-ml aliquots of the column fractions on aluminum planchets, drying, and counting in the gas-flow counter. In the majority of the fractions this volume gave a plate which could be considered to be infinitely thin. Because of the large number of fractions assayed no attempt was made to correct for sample absorption in those fractions not drying to an infinitely thin layer.

Scintillation counting was used to determine the recovery of the administered radioactivity from the urine. This method was also used to determine the radioactivity in a particular sample, when accuracy greater than that provided by uncorrected gas-flow counting was required. The scintillation fluid used for counting the radioactivity in these aqueous samples was that of Bray (18). Quenching as a result of the color or high salt content, or both, of a particular sample was corrected for by adding to a triplicate sample 0.2 to 0.5 ml of the scintillation fluid containing a known amount of thiamine-C¹⁴.

RESULTS

A typical pattern of the radioactive metabolites of thiamine-C¹⁴ eluted from an Amberlite CG-50 column is shown in figure 1. The pattern of eluted radioactivity is representative of a pooled de-salted urine sample from 10 rats, each of which received an intraperitoneal injection of 100 µg of thiamine-C¹⁴ daily for 30 days prior to collection. Five major peaks of radioactivity are evident. Peak no. 1 is only slightly cationic in character since it was readily eluted. Peaks no. 2 through 5 contain compounds of increasing basicity. Peak no. 5 contains thiamine-C¹⁴ as well as some 10 other metabolites of thiamine-C¹⁴. The activities in the various

⁷ Mallinckrodt Chemical Works, St. Louis, Missouri.
⁸ AG-1-X8, Mo-Rad Laboratories, Richmond, California.

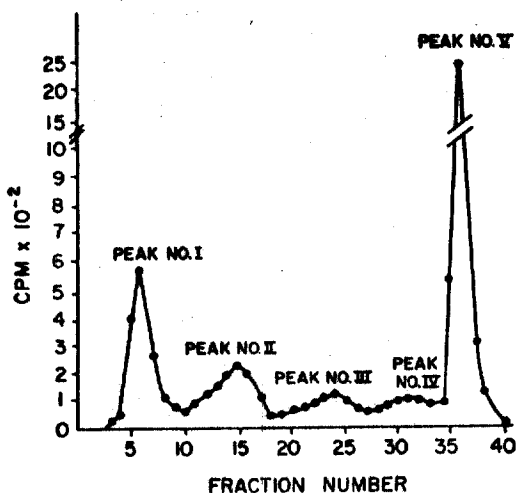


Fig. 1 Column chromatography of desalted rat urine. Exchanger, Amberlite CG-50 (200 400 mesh) in the H^+ form, 1×40 cm. Flow rate 60 ml/hour, fraction size 12 ml. The activity in counts per minute as determined by gas-flow counting is that of 0.50-ml aliquots of the 12-ml fractions.

peaks were found to vary both qualitatively and quantitatively depending upon the amount of thiamine- C^{14} administered, the state of thiamine nutrition of the animal, and the length of time the animal had been receiving radiothiamine. Peaks 1 and 2 contain a greater percentage of the total radioactivity in rats injected with thiamine- C^{14} for a longer period of time than in rats injected for a shorter period. A time delay of about 2 days in the appearance of these 2 peaks has also been observed in animals which had just started receiving thiamine- C^{14} .

It was necessary to subject peak no. 1 to further ion exchange chromatography on Dowex-1-chloride prior to paper chromatography. The pattern of radioactivity from such a column is shown in figure 2. This pattern represents the radioactivity of a pooled desalted 24-hour urine from 10 rats, each of which received intraperitoneal injections of 100 μ g of thiamine- C^{14} /day for 39 days prior to collection. This procedure separated the bulk of the radioactivity in peak 1 from contaminating solid material so as to permit satisfactory paper chromatography. For this purpose the main radioactive peak (fractions 15-17) was reduced to dryness in the manner previously described.

Ascending paper chromatography of the lyophilized samples of the 5 radioactive peaks shown in figure 1 was carried out for 18 hours on acid-washed Whatman 3 MM paper. The paper was then dried and a radioautogram prepared by placing it in contact with Eastman "No-screen" X-ray film for an average of 10 days. The pattern of radioactive metabolites obtained and their average R_f values is schematically represented in figure 3. In all, 22 metabolites of thiamine- C^{14} were detected in urine of rats by radioautography. It is probable that this is a minimal figure since, with one exception, no attempts were made to rechromatograph individual radioactive bands in another solvent to determine whether they contained more than one radioactive component. It is also possible that a film exposure time greater than that used might have revealed the presence of additional minor metabolites. Metabolite no. 16, figure 3, is thiamine. The percentage of the radioactivity in the urine represented by thiamine- C^{14} was determined by cutting the area corresponding to thiamine from the chromatogram, eluting the radioactivity with distilled water, and counting in the Packard Tri-Carb scintillation counter. The

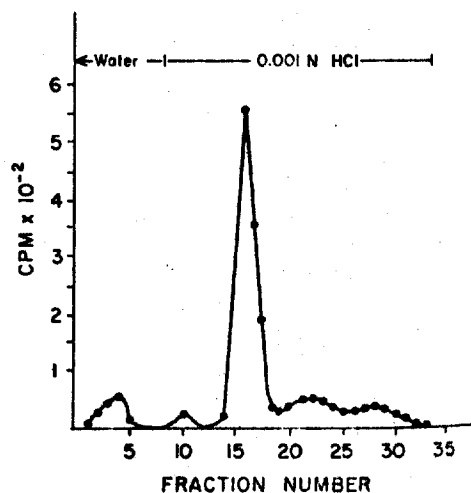


Fig. 2 Ion exchange chromatography of peak no. 1 from the Amberlite CG-50 column (see fig. 1). Exchanger, Dowex-1-chloride (100-200 mesh), 1×20 cm. Flow rate 60 ml/hour, fraction size 12 ml. The activity in counts per minute as determined by gas flow counting is that of 0.50-ml aliquots of the 12 ml-fractions.

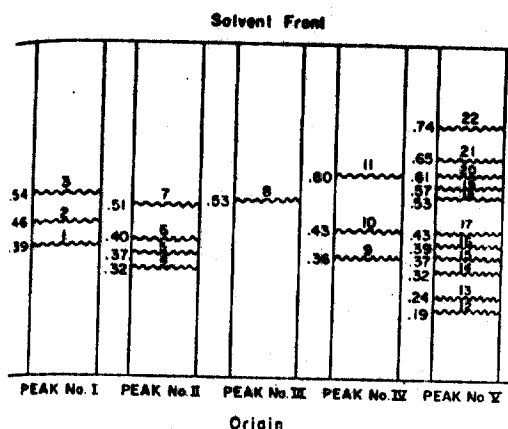


Fig. 3 Schematic representation of the radioautograms of the radioactive peaks from an Amberlite CG-50 column of the desalted urine of rats receiving thiamine- C^{14} (see fig. 1). Solvent n -propanol/water/1 M acetate buffer pH 5.0 (70/20/10), by volume (22). Ascending technique for 18 hours.

thiamine- C^{14} excreted in a 7-day period was found to represent 48 to 51% of the total activity of the urine collected from 10 rats injected daily with 100 μ g of thiamine- C^{14} for 40 days or more. However, this proportion of the total radioactivity as thiamine- C^{14} is characteristic only at an intake level of 100 μ g/day since the percentage of the total urinary radioactivity which is thiamine- C^{14} will vary in proportion to the amount of thiamine administered.

There was a significant variation of the R_f values from one radioautogram to the next. The range of R_f values is shown in table 1. Metabolite no. 16 in table 1 is thiamine- C^{14} . The R_f value of authentic thiamine- C^{14} when spotted from distilled

TABLE 1
Range of R_f values obtained for the metabolites of radiothiamine shown in figure 3

Metabolite no.	Range of R_f values	Metabolite no.	Range of R_f values
1	0.35-0.42	12	0.18-0.21
2	0.43-0.50	13	0.21-0.27
3	0.50-0.57	14	0.31-0.34
4	0.30-0.36	15	0.34-0.41
5	0.33-0.39	16	0.35-0.45
6	0.36-0.43	17	0.39-0.48
7	0.50-0.54	18	0.53-0.56
8	0.53-0.54	19	0.56-0.61
9	0.36	20	0.59-0.61
10	0.43-0.43	21	0.64-0.65
11	0.59-0.61	22	0.74

water solution is 0.38. Metabolites no. 9 and 22 were detected only once on a radioautogram which had been exposed to X-ray film for approximately 3 weeks. The remaining metabolites were detected a minimum of 3 times on separate radioautograms of separate urine collections. The variation of R_f values may be ascribed to 2 factors, both of which are dependent upon the amount of urine placed on the chromatogram. The first is the amount of brown oily urinary pigment contained in each radioactive peak from the Amberlite CG-50 column. This tends to increase the R_f values of the metabolites apparently in direct proportion to its concentration. The second factor is the amount of solid material in the sample to be chromatographed. These solids of indeterminate nature tend to decrease R_f values and make the radioactive bands more diffuse. This effect was particularly evident when the Amberlite CG-50 column peaks obtained from the pooled 24-hour urine of 20 or more rats were chromatographed on paper.

An alternate method of developing the Amberlite CG-50 column consisted of elution with water for 200 ml followed by elution with pyridine/acetic acid/water (7.5/1/91.5, by volume). The pattern of radioactivity obtained by use of this method is shown in figure 4. The advantage of this elution procedure is that thiamine- C^{14} can be separated from all but one of its radiometabolites. A schematic representation of radioautographs of the 4 radioactive peaks obtained by this elution procedure is shown in figure 5. For paper chromatography it was necessary to subject peak no. 1, figure 4, to the same Dowex-1 treatment described for peak no. 1, figure 1.

Because of the similarity of the initial elution procedure, the radiometabolites of peaks no. 1 and no. 2, figure 5, are of the same number and approximate R_f values as the same peaks in figure 3. Radioactive bands 8, 9, 10, and 11 from figure 3 are now a part of peak no. 3 in figure 5. Considering the similarity in R_f values, radioactive bands 9 and 15 in figure 3 are probably combined to form radioactive band no. 11 in figure 5. In addition, radioactive bands 10 and 17 as well as 11 and 20 from figure 3 are probably combined

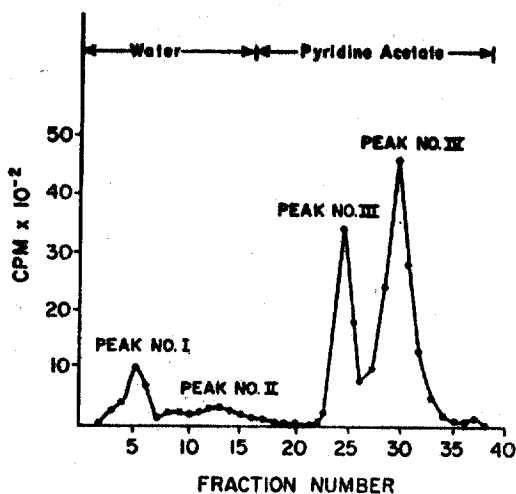


Fig. 4 The chromatography of desalted urine using a pyridine/acetate eluent. Exchanger, Amberlite CG-50 (200-400 mesh) in the H^+ form, 1×40 cm. Flow rate 60 ml/hour, fraction size 12 ml. The pattern of radioactivity shown is representative of that found in a pooled desalted 24-hour urine sample from 10 rats each of which received 200 μ g of thiamine- C^{14} by way of 2 intraperitoneal injections of 100 μ g 12 hours apart. The activity in counts per minute as determined by gas-flow counting is that of 0.50- μ l aliquots of the 12-ml fractions.

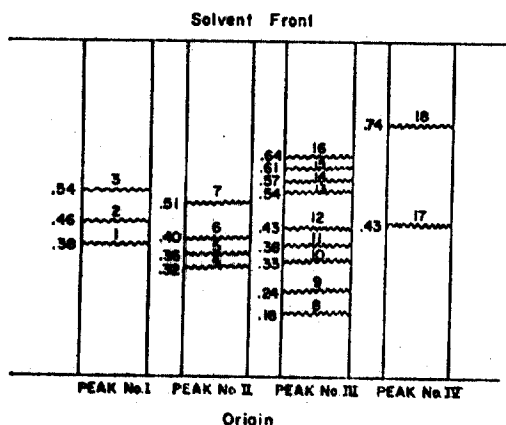


Fig. 5 A schematic representation of the radioautograms of the radioactive peaks from an Amberlite CG-50 column of desalted urine eluted with pyridine/acetate (see fig. 4). Solvent for paper chromatography *n*-propanol/water/1 M acetate buffer pH 5.0 (70/20/10, by volume) (22). Ascending technique for 18 hours.

to form bands 12 and 15, respectively, in figure 5, metabolite no. 17 in figure 5 is thiamine- C^{14} .

A New Zealand rabbit weighing 2.93 kg was injected intraperitoneally with 1.265

mg (1.11 μ g) of thiamine- C^{14} on the first day and with 1.000 mg (0.88 μ g) on 3 succeeding days. An aliquot consisting of one-third of the volume of each of the 3-day urine collections was examined chromatographically for breakdown products of thiamine- C^{14} .

The qualitative pattern of radioactivity eluted from CG-50 columns was similar to that shown in figure 1 for the rat. Radioautographs of the CG-50 peak 1 from rabbit urine showed radioactive bands coinciding with bands 1 and 2, figure 3. Peak 5 radioautographs from rat and rabbit urine are compared in figure 6. Three radioactive bands detectable in rat urine were not observed in rabbit urine. Two obvious quantitative differences were evident. (1) The radioactive band with an R_f of 0.17 in rabbit urine contained a greater percentage of the total activity in the peak than did the band in rat urine with an R_f of 0.19. The latter band was actually absent from some chromatograms. (2) The rat urine band with an R_f of 0.61 was present in much larger quantity than the corresponding band in rabbit urine. It should be emphasized, however, that a strict comparison is not entirely valid since the labeled thiamine was administered to the rabbit for only 4 days and his tissue stores were not as

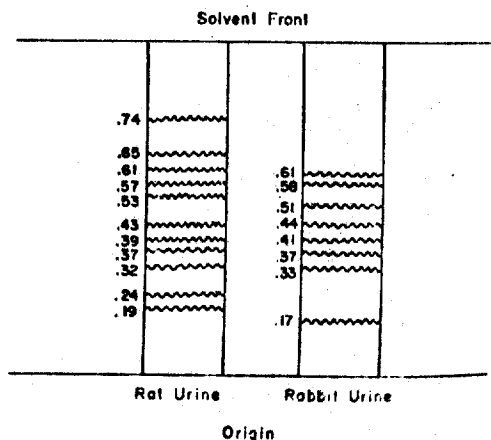


Fig. 6 A schematic comparison of the radioautograms of peak no. 5 from an Amberlite CG-50 column of desalted rabbit urine with the corresponding peak from rat urine. The solvent in both cases was *n*-propanol/water/1 M acetate buffer pH 5.0 (70/20/10, by volume) (22). Ascending technique for 18 hours.

completely labeled as those of the rats studied. Because of the small amount of radioactivity present, peaks 2, 3, and 4 were not subjected to paper chromatography.

It became evident early in this study that for the radioautographic data to have any validity, a determination of the stability of thiamine in urine would have to be undertaken. To this end, a group of 14 Sprague-Dawley rats were fed a thiamine-free diet for one week. Then, 7 of the rats were given daily injections of 30 μ g of thiamine- C^{14} . Separate urine collections were made from the injected and noninjected animals. An amount of thiamine- C^{14} equal to or in some cases greater than that injected was added to the pooled urine of the noninjected animals. To correlate easily the R_f values of any compounds resulting from the possible breakdown of the thiamine- C^{14} which was added to the urine with those resulting from the injection of thiamine- C^{14} , the Amberlite CG-50 peaks of radioactivity of both urines were chromatographed on the same piece of filter paper.

In the first experiment the urine containing the added thiamine- C^{14} was adjusted to pH 8.6 and incubated at 37° for 3 hours. Subsequent ion exchange chromatography of the desalted urine indicated the presence of a small amount of radioactivity corresponding to peak no. 1, figures 1 and 4, with the remainder of the activity corresponding to peak no. 5 in figure 1. Radioautographs of these peaks from the urine samples of both groups of animals revealed 2 breakdown products of the thiamine- C^{14} as a result of incubation with the urine. The R_f values of these breakdown products were similar to those of band nos. 1 and 18 in figure 3. The radioactivity data from the Amberlite CG-50 column indicated that the breakdown product with an R_f value similar to metabolite no. 1, figure 3, represented about 2.5% of the total activity incubated with the urine. No attempt was made at quantitating the amount of the breakdown product corresponding to band no. 18, figure 3, but from the darkening of the film it appeared to be quantitatively as great as the metabolite with a similar R_f value from the urine of the injected rats. Two

additional radioautographs under the same conditions of incubation gave results almost identical to those described. In a further study of thiamine stability, the pooled urine of the noninjected animals was adjusted to pH 9.0, 350 μ g of thiamine- C^{14} added and the urine incubated at room temperature for 24 hours. Subsequent column chromatography gave the same pattern of activity as in the first incubation study except that the radioactivity of the early peak made up about 10% of the total. Comparative radioautography with the urine of the injected rats again indicated the presence of bands which were similar in R_f to bands nos. 1 and 18, figure 3, plus an additional radioactive band with an R_f value similar to that of band no. 3, figure 3. Finally, thiamine- C^{14} was added to the urine collected without acidification. At the end of the 24-hour collection period the pH of the pooled urine was measured and found to be 7.6. Subsequent column chromatography followed by radioautography showed the presence of only a trace of a breakdown product with an R_f similar to that of radioactive band no. 18, figure 3. This experiment was repeated under the same conditions except that the pooled 24-hour urine was then incubated for 4 hours at 37° and was permitted to stand at room temperature for an additional 20 hours. The subsequent radioautograph again revealed the presence of only a trace amount of a material with an R_f value similar to band no. 18, figure 3.

DISCUSSION

A minimum of 22 different products of C^{14} -pyrimidine-labeled thiamine were detected in the urine of rats. Although the bulk of the evidence indicates otherwise, the possibility exists that 3 of the products were formed as a result of breakdown of the radioactive thiamine in the urine.

It is difficult to believe that each of these 20 or more metabolites is a separate chemical species. What is more probable is that a portion of these metabolites represent products of the action of the various detoxification enzymes of a lesser number of metabolites of the thiamine molecule. For example, the glucuronide, phosphate, or sulfate ester of the same metabolite

might be formed. Another source could be the formation of a series of mixed disulfides of thiamine. In future work, the most promising way of assigning a general structure to these metabolites would be by using 2 forms of thiamine-C¹⁴, one of which is labeled in the pyrimidine ring, and another labeled in the thiazole ring. Using this approach it should be possible, using the purification and separation methods described, to distinguish between metabolites that are some form of pyrimidine moiety, some form of the thiazole moiety, and some form of both the pyrimidine and thiazole moieties.

The thiamine stability studies indicated that thiamine is quite stable for extended periods of time at room temperature at the normal pH of urine. These results are in agreement with those obtained by Melnick and Field (19). These workers reported that for any breakdown of thiamine to occur in urine incubated at 37.5° for 6 hours, it was necessary to adjust the pH to 9.0 or higher. Our results demonstrate that there is a negligible breakdown of thiamine in urine kept at pH values less than 8.6. At or above pH 8.6, however, some breakdown occurs leading to the appearance of 2 or 3 different compounds depending upon the pH.

The mechanism by which thiamine is broken down remains to be investigated. The relative stability of thiamine-C¹⁴ in urine indicates that the bulk of the radio compounds are true metabolic products. It appears that there are 2 possible sources of these metabolites: 1) compounds formed as a result of thiamine acting as a coenzyme, as for example, the compound 2-(1-hydroxyethyl)-thiamine pyrophosphate which is formed as a consequence of the participation of thiamine pyrophosphate in the pyruvate decarboxylase reaction (20), and 2) products of the actions of degradative enzymes such as the thiaminases on thiamine. In any event, further work on the structure of the metabolites of thiamine should give valuable information concerning the reactions involved in providing the rather large number of metabolites of the thiamine molecule.

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Studies of Thiamine Metabolism in the Rat

II. ISOLATION AND IDENTIFICATION OF 2-METHYL-4-AMINO-5-PYRIMIDINECARBOXYLIC ACID AS A METABOLITE OF THIAMINE IN RAT URINE^{1,2}

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ABSTRACT A compound isolated from the urine of rats receiving thiamine-C¹⁴ has been identified as 2-methyl-4-amino-5-pyrimidinecarboxylic acid. Column and paper chromatographic evidence indicates this same compound is also present in the urine of germfree rats and in rabbit and human urine.

In a previous publication (1) the detection of 22 different metabolites of thiamine-C¹⁴ in the urine of rats receiving daily intraperitoneal injections of labeled vitamin was described. Subsequently, attempts have been made to identify the major pyrimidine metabolites of thiamine in mammalian urine.

It would be expected that some form of the pyrimidine moiety of thiamine would be present in the urine of the rat and rabbit because it is well established that the thiazole moiety of thiamine is excreted in the urine of these species (2-4). One form of the pyrimidine moiety which might be expected to occur in urine is 2-methyl-4-amino-5-hydroxymethylpyrimidine (HMP). This compound would be generated as a result of hydrolytic cleavage of the covalent bond between the methylene bridge and the thiazole ring and is known to be a product of the action of a specific bacterial thiaminase (5). Although Kawasaki and Okada (6) have reported the isolation of HMP from human urine, repeated attempts in our laboratories to detect this compound in the urine of rats receiving pyrimidine-C¹⁴-labeled thiamine were unsuccessful. The use of HMP-C¹⁴ as an intermediate in our synthesis of thiamine made it convenient to look for HMP in human urine by using it as a marker. In 2 separate experiments, HMP-C¹⁴ was added to human urine and re-isolated by the column and paper chromatographic methods used in the isolation of metabolites of thiamine-C¹⁴ from rat

urine. Since no decrease in the specific activity of the added HMP-C¹⁴ was observed in either case, we must conclude that this compound does not normally occur in rat or human urine. The failure to detect HMP in mammalian urine led us to investigate its possible conversion into another product. The results of this study, which are recorded here, provide evidence that 2-methyl-4-amino-5-pyrimidinecarboxylic acid is a major product of the mammalian catabolism of thiamine.

EXPERIMENTAL PROCEDURE

The methods for determining radioactivity as well as the procedures for isolation and detection of the metabolites of thiamine-C¹⁴ have been described previously (1).

In a study designed to determine the amount of HMP-C¹⁴ converted to C¹⁴O₂, rats were placed in a standard respiration chamber and the expired carbon dioxide collected by drawing the expired air through 175 ml of 10% sodium hydroxide. The trapped carbon dioxide was precipitated as barium carbonate by addition of 20% barium chloride to a suitable aliquot of the sodium hydroxide. The barium carbonate precipitate was collected by cen-

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² A portion of the data was taken from a thesis submitted by R. A. Neal to the faculty of the Graduate School of Vanderbilt University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

trifugation and washed twice by resuspension in 1% ammonium chloride and in turn in water followed in each case by centrifugation. The barium carbonate was then dried in an oven at 110° for 12 hours. The activity of the dried barium carbonate was determined by the method of Ott et al. (7).

The animals and animal diets, as well as the procedures for collection of the urine have been described previously (1). In studies with germfree rats¹ the animals were fed a sterile thiamine-deficient diet similar to that described previously except that cornstarch replaced the sucrose and the succinylsulfathiazole. Six germfree rats were individually housed in wire-screen cages which were set over a funnel to allow urine collection. The duration of this experiment was 7 days. It was not practical to collect the urine daily during this study so the pooled 7-day urine sample was collected in toto on termination of the experiment.

The chemical synthesis of 2-methyl-4-amino-5-pyrimidinecarboxylic acid was accomplished by the hydrolysis of 2-methyl-4-amino-5-cyanopyrimidine with sodium hydroxide. The experimental details are as follows: 250 mg of 2-methyl-4-amino-5-cyanopyrimidine² which had been recrystallized from absolute methanol, was hydrolyzed with 5 ml of 10% sodium hydroxide on a boiling water bath. Termination of the hydrolysis was indicated by the cessation of the evolution of ammonia (about 10 minutes). The reaction mixture was acidified with 5 N hydrochloric acid and the pH of the solution adjusted to 4.5 at which time a copious precipitate formed. The resultant compound was twice recrystallized from a mixture of methanol/ethanol/water (40/40/20, by volume). Elementary analysis³ of the synthesized compound was as follows: calculated for $C_5H_6N_4O_2$: C, 47.03; H, 4.61; N, 27.45; found: C, 46.98; H, 4.48; N, 27.35.

RESULTS

Since attempts at detecting HMP in the urine of rats or man were unsuccessful, the possibility that it was extensively degraded was investigated by measuring the expiration of $C^{14}O_2$ following the intraperitoneal injection of HMP- C^{14} into rats. In

the initial experiment 60 μ g (0.113 μ c) of HMP- C^{14} were injected into a rat which had been fed a stock diet ad libitum. The expired CO_2 was collected in 3-hour increments for 12 hours following the injection. The radioactivity in the precipitated barium carbonate amounted to 1.26% of that administered. In a second experiment, in which 60 μ g of HMP- C^{14} were injected into a rat that had been fasted for 24 hours, 1.27% of the administered radioactivity appeared as $C^{14}O_2$ in the 12 hours following the injection.

Because these experiments indicated that injected HMP- C^{14} was not extensively degraded, the compound was again injected intraperitoneally into rats and the urine examined for metabolites by the methods used in the studies with thiamine- C^{14} (1). The pattern of radioactivity resulting from ion exchange chromatography of the desalted urine of rats receiving HMP- C^{14} is shown in figure 1. Because of the large amount of contaminating material, it was necessary to subject the pooled fractions of peak no. 1, figure 1, to further ion exchange chromatography on Dowex-1-C1 prior to paper chromatography. The pattern of radioactivity resulting from rechromatography of this peak is shown in figure 2. The pattern of activity (fig. 2) resembles that obtained by rechromatography of the corresponding peak for the Amberlite CG-50 column of the desalted urine of rats receiving thiamine- C^{14} .

Fractions 16 and 17, figure 2, as well as fractions 23 to 25 (peak no. 2) and 34 to 37 (peak no. 3), figure 1, were reduced to dryness and chromatographed (ascending) on acid washed Whatman no. 3 MM paper, for 18 hours in *n*-propanol/water 1 M acetate buffer pH 5, (70/20/10, by volume) (8). The subsequent radioautograph of the radioactive peak of figure 2 showed the presence of 3 radioactive bands with R_f values of 0.39, 0.46, and 0.54. These 3 bands corresponded in both R_f value and characteristic shape with 3 radioactive bands isolated in identical

¹ The studies with germfree rats were made possible through the generosity of Dr. Floyd Daft and Mr. E. R. McDaniel of the National Institutes of Arthritis and Metabolic Diseases, National Institutes of Health.

² Aldrich Chemical Company, Inc., Milwaukee, Wisconsin.

³ Clark Microanalytical Laboratory, Urbana, Illinois.

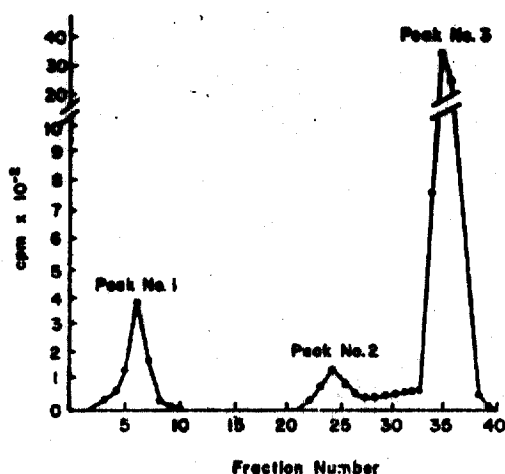


Fig. 1 The chromatography of the urine from rats receiving 4,7- C^{14} -2-methyl-4-amino-5-hydroxymethylpyrimidine (C^{14} -HMP). Exchanger, Amberlite CG-50 (200-400 mesh), 1.0×40.0 cm, in the H^+ form. Flow rate 60 ml/hour, fraction size 12 ml. The activity in counts per minute as determined by gas-flow counting is that of 0.5-ml aliquots of the 12-ml fractions.

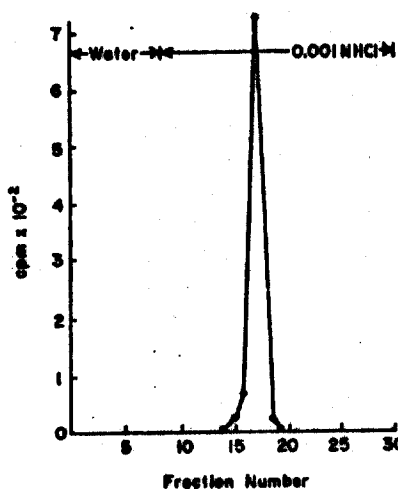


Fig. 2 Ion exchange chromatography fractions 5-7, Figure 1, on Dowex-1-chloride (100-200 mesh), 1×30 cm. Flow rate 60 ml/hour, fraction size 12 ml. The activity in counts per minute as determined by gas-flow counting is that of 0.5 ml aliquots of the 12-ml fractions.

fashion from the urine of rats receiving thiamine- C^{14} .

The radioautograph corresponding to peak no. 2, figure 1, indicated the presence of a radioactive band with an R_f value of

0.59. The R_f value for the radioactive band resulting from radioautography of the corresponding peak of radioactivity from an Amberlite CG-50 column of the urine of rats receiving thiamine- C^{14} is 0.53.

The radioautograph of peak no. 3, figure 1, revealed the presence of 2 radioactive bands. One of these, with an R_f value of 0.56 was identified as unchanged HMP- C^{14} on the basis of its ultraviolet spectrum. The second band had an R_f of 0.39 which is also the R_f value of thiamine in the same solvent system. This band, however, did not fluoresce when sprayed with ethanol/10% sodium hydroxide/2.5% potassium ferricyanide (2/1/0.05, by volume), a mixture which converts thiamine and its esters into their corresponding thiochrome derivatives. Unfortunately, this radioactive band was in an area on the paper chromatogram which contained a large amount of a brown pigment which may have quenched traces of thiochrome fluorescence.

Peak no. 1, figure 1, accounted for 12 to 18% of the total activity eluted from 3 separate columns and the radioactive band in this peak with an R_f of 0.46 contained 73 to 82% of the total radioactivity in the peak. The bands of radioactivity with R_f values of 0.39 and 0.54 contained about equal amounts of the remaining activity. The 3 radioactive compounds in the corresponding peak from the urine of rats receiving thiamine- C^{14} exhibited about the same quantitative ratio.

Peak no. 2, figure 1, contained 5 to 6% of the activity eluted from the column with peak no. 3 accounting for the remainder. Approximately 99% of the radioactivity in peak no. 3 was due to HMP- C^{14} .

When peak no. 1, figure 1, was isolated from the urine of rats receiving 100 μ g/day of thiamine- C^{14} , it was found to contain 6 to 12% of the total activity eluted from the column. Thus each rat excreted 5 to 9 μ g of the injected radioactive thiamine in the form of 3 acidic metabolites which behaved the same on column and paper chromatography as compounds resulting from the metabolism of injected HMP- C^{14} .

The acidic nature and quantitative importance of the radioactive band with an R_f value of 0.46 isolated from the urine

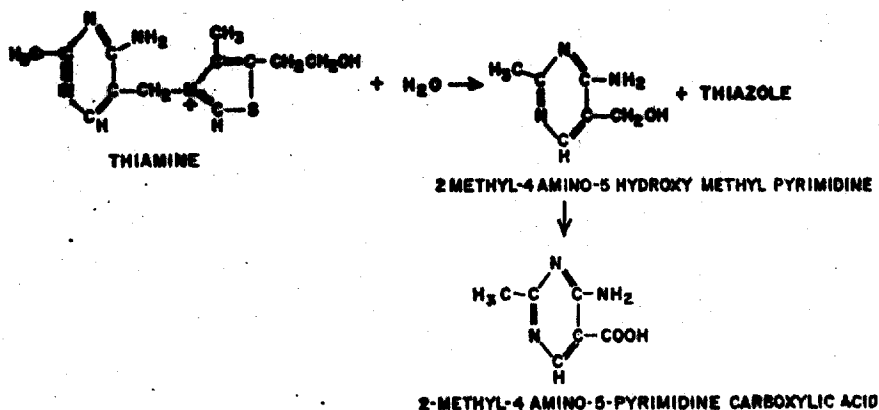


Figure 4

a healthy human volunteer (male, Caucasian, age 65 years) ingested 1 mg (0.88 μ c) of thiamine- C^{14} daily for 5 days. A portion of the 24-hour urine samples was analyzed for the presence of the unknown compound using the same techniques described for rat urine. Although the specific activity of the urine was low, the column and paper chromatographic evidence indicated strongly that the compound in question was also present in human urine. As reported in a previous publication (1), a compound corresponding to PCA is also present in rabbit urine.

DISCUSSION

The evidence presented indicates that an acidic compound excreted in the urine of rats receiving thiamine- C^{14} has the structure 2-methyl-4-amino-5-pyrimidinecarboxylic acid. Column and paper chromatographic evidence demonstrates that this compound is also present in rabbit and human urine as well as in the urine of germfree rats. Quantitatively it represents from 5 to 10% of the total activity excreted in the urine of rats receiving 100 μ c of thiamine- C^{14} daily.

Although no data have been obtained on the mechanism of the derivation of this compound from thiamine, a logical sequence would be hydrolytic cleavage (enzymatic or nonenzymatic) of the covalent bond between the methylene carbon and the thiazole ring nitrogen to yield 5-(2-hydroxyethyl)-4-methylthiazole and HMP followed by enzymatic oxidation of the HMP

to PCA. Such a provisional scheme is shown in figure 4. The existence of HMP as an intermediate is suggested by the observation that this compound, when injected, is partially converted to PCA. Evidence for the formation of a carboxyl group from a 5-hydroxymethyl group of a pyrimidine has been reported by Fink et al. (9). These workers reported chromatographic evidence for the formation of uracil-5-carboxylic acid from thymine by rat liver slices with 5-hydroxymethyl uracil being a possible intermediate. The same workers have also reported similar observations in *Neurospora crassa* with the addition that 5-formyl uracil was also identified as a metabolic product.⁶ Although it is possible that a similar oxidative sequence might apply to the metabolism of the pyrimidine moiety of thiamine, we have not examined this possibility.

ADDENDUM

Two reports bearing on this subject have recently come to our attention. Shintani (10) has reported the detection of PCA in the urine of the rabbit after injection with HMP. His absorption spectra closely resemble those recorded here. Matthies and Peters (11) have recently studied the cytostatic effects of HMP derivatives in mice. PCA was found to be considerably less toxic than HMP, suggesting that it may serve as a detoxification product of the latter compound.

⁶ Fink, R. M., and K. Fink 1963 Biological conversion of thymidine to non-methylated pyrimidines. Federation Proc., 21: 377 (abstract).

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B-Complex and Enriched Flour as Causes of Atopic Dermatitis

To the Editor.—Cases of hypersensitivity to B-complex preparations have been reported, including urticaria, angioneurotic edema, and contact dermatitis. After intravenous injection, anaphylactoid reactions have occurred, including some which were fatal.¹

The current widespread use of ascorbic acid and of preparations containing high doses of the other water-soluble vitamins makes the following case of some interest.

Report of a Case.—A woman, aged 50 years, developed a severe pruritic dermatitis, covering body and limbs, but not the face. This disturbing eruption showed no uniform lesions: some were scaly, some vesicular and erythematous, and some areas showed lichenification. The palms of both hands, the legs, and the nuchal region were particularly affected. The eruption was thought at first to be possibly fungal in origin, but cultures proved negative. Various other tentative diagnoses included nummular eczema, erythema multiforme, and neurodermatitis.

Since the patient had received no medication for some time, an allergic reaction to detergents or enzymes was considered. On repeated questioning about medication, the only substances mentioned as ingested besides food were "B-complex capsules," at first not considered as "medication" by the patient. The dose used was one to two capsules daily of a "therapeutic" combination obtainable at any drug store.

Discontinuance of these capsules resulted in cessation of all symptoms within 48 hours. This history might not be unusual, except for some sequelae. The eruption recurred off and on to a much lesser degree, even though no vitamins were taken. Exploration revealed that the patient could provoke the dermatitis by eating several slices of white bread made with enriched flour (thiamine, riboflavin, and niacin added), or anything else baked with such flour. This represents probably an unusual degree of sensitivity, yet it is possible that some obscure types of dermatitis may be triggered by "enriched" foods, leaving the treating physician puzzled as to the causative agent, even when sensitization is suspected.

Communications regarding similar experiences would be needed to assess the frequency of such effects.

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COR et VASA 4 (4) : 289-295, 1962

CONCERNING THE SPECIFIC SENSITIVITY OF THE HEART TO THE ADMINISTRATION OF THIAMINE

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Many and varied symptoms of disturbances of cardiac function with thiamine deficiency have been described in detail in the literature. It is known that one form of B₁ avitaminosis is cardiac failure, involving marked hypotension and dystrophic changes in the myocardium (1). It is further known that the heart is excessively sensitive to various anti-thiamine factors (2). In many investigations of thiamine metabolism in diseases of the heart and vessels (3-5) the attention of the authors was devoted to the origin of the signs of thiamine deficiency. The results were strikingly contradictory. In our previous observations (6-10) it was shown that in essential hypertension the blood serum contains raised amount of free thiamine and thiochrome, activated transketolase and thiamine dehydrase. This was a basis for the assumption that essential hypertension is characterised by hypervitaminosis B₁ rather than the reverse. In essential hypertension biochemical changes were found which are particularly interesting with reference to the marked sympathetic-like effect of thiochrome on the isolated frog heart (11) and also in relation to the synergistic effect of thiamine in cholesterol synthesis.

We have studied the effect of experimental avitaminosis and hypervitaminosis B₁ on the heart and some metabolic processes in the pigeon myocardium.

METHODS

All of the pigeons in the experiment were maintained on a standard diet of husked rice for 20 to 30 days. The avitaminosis group received only rice. On the average, from the 8th to the 10th day they were forced fed and at about the 24th day they developed signs of avitaminosis and polyneuritis. From this period on, they were sacrificed, one bird from each group, birds being selected from the degree of polyneuritis in the avitaminosis group (opisthotonus, stasis). In some experiments, tissue fluid from fresh water molluscs was added to the diet in order to accelerate the development of avitaminosis symptoms. The presence of thiaminases in this fluid accelerated the development of specific polyneuritis on the average by 5-10 days.

The group which we considered normal received the same diet, but in addition each

bird received 10 μ g. of thiamine daily. In a specially arranged control experiment, blood and tissue of these pigeons were compared with samples from birds on a normal mixed grain diet. The level of pyruvic acid in the blood, and transketolase activity in erythrocytes, liver, heart, brain, plasma and muscle were compared in paired animals. This enabled us to state that a dosage of 10 μ g. of thiamine daily completely filled the animals' requirements. The rice diet was only used in order to be able to rule out other factors in comparing the various groups, since they all received the same dietary basis, with the exception of the thiamine content.

The hypervitaminosis group received 100-150 μ g. of thiamine daily added to the same basic diet.

For objective control purposes, in part of the birds the livers were analysed quantitatively for thiamine diphosphate, using the manometric method of Westenbrink, as modified by Engelhardt and Kanopkaite (13) with yeast apocarboxylase. Mean values of thiamine diphosphate in μ g./g. of fresh liver were as follows: avitaminosis: 1.23, controls: 6.16, hypervitaminosis: 12.44.

The pigeons were sacrificed by decapitation, and after cooling of the body segments of muscle (apex of the heart) were removed and ground in a glass homogenizer. In the homogenate the following activities were estimated: transketolase (14), thiamine dehydrase (15), aldolases and transaminases (16), cholinesterase (17), along with the quantities of phospholipids and SH groups (18). 85 animals were used in all.

RESULTS AND EVALUATION

Macroscopic differences found at post mortem between the avitaminosis and normal group will not be described, since these are already well known. It was surprising that there was a frequent and marked difference between the controls and the hypervitaminosis group. In the latter case we observed in some cases excessive fat deposition in the subcutaneous tissues, omentum, and, of particular importance, about the heart itself. At times the hearts of the hypervitaminosis group were encased in a massive fat envelope, with a "wreath" about the organ between the atria and ventricles.

In avitaminosis, this cardiac fat deposit was always lacking, and the myocardium was exhausted and weak. On the other hand, in the hypervitaminosis group the myocardium was firmer and often thicker than normal. These macroscopic impressions were confirmed by heart weights in the three groups (Tab. I). Attention should be called to the fact that the scatter in organ weights of the normal animals and in the hypervitaminosis group was the same, so that the increased relative weight of this organ further stresses the specific role of thiamine in causing this difference.

Of greatest interest were observations on the particularities of thiamine metabolism in the myocardium. If there were marked macroscopic effects of hypervitaminosis B₁ on the heart, there must be metabolic changes to this in terms of the metabolism of thiamine *per se*. In determining the activity of transketolases we had a view on the one hand of the activity of proteidisation of thiamine diphosphate as a co-enzyme, and on the other hand of the activity of the pentose phosphate cycle, i. e. oxidative breakdown of carbohydrates. The results (Tab. I) directly show that hypervitaminosis leads to a marked activation of oxidative

breakdown of carbohydrates: the amount of sedoheptulose-7-phosphate was significantly raised.

Of particular interest was the fate of free thiamine. As it has already been shown there is direct relation between the thiamine oxidation and resynthesis of sympathines, which play an important role in the regulation of trophic functions and a general role in cardiovascular activity.

The rate of resynthesis of sympathine can be judged from the thiamine dehydrase reaction, in which thiamine is oxidised to thiochrome, and at the expense of the latter dihydroadrenaline is converted to adrenaline. The results (Tab. I) show that thiamine dehydrase activity is directly

Tab. I. Metabolic changes in the myocardium of pigeons with various dosages of thiamine.

		Hypo.	Control	Hyper.
Transketolase (μ M sedoheptulose-7-phosphate per gr./hr.)	M t P	3.13 8.16 <0.001	5.85 — —	8.63 6.16 - 0.001
Thiamine dehydrase (% of control)	M t P	159 6.80 <0.001	194 — —	262 5.95 <0.001
Aldolase (av. values)	M t P	18.5 2.56 0.02	19.5 — —	21.0 2.0 0.05
Transaminase (rel. u./gr.)	M t P	— — —	90,000 — —	138,000 5.9 - 0.001
Cholinesterase (av.)	M t P	— — —	52 — —	48 0.56 > 0.5
SH groups (μ Mol/gr.)	M t P	— — —	6.5 — —	7.3 2.0 0.1 - 0.5
Relative heart weight (%)	M t P	1.14 11.6 <0.001	1.36 — —	1.19 5.59 <0.001
Cholesterol (mg.%)	M t P	205 3.53 - 0.01	175 — —	199 2.50 - 0.02
Phospholipids (mg.%)	M t P	2132 2.88 - 0.01	3104 — —	2462 1.90 0.05 - 0.1

dependent on the uptake of thiamine. It should be stressed that while in the presence of adrenaline and its oxidation products pigeons from all three groups showed a marked thiamine dehydrase activity, without sympathine in the avitaminosis group and under normal, control conditions, the opposite occurred, i.e. a decline in thiamine dehydrase activity. Only in the hypervitaminosis group does the conversion of thiamine to thiochrome have a significance *per se*, not related to sympathine. This latter fact is deserving of particular attention in connexion with the known activity of thiochrome *per se*, similar to sympathine (8, 9).

In order to get some idea of the state of anaerobic breakdown of carbohydrate, N. K. Lukashik studied in our laboratory aldolases in homogenates of pigeon heart. As with transketolases, the activity changed as a function of thiamine uptake. The results are presented in terms of protein content as related to the controls.

In determining the activity of glutamic aspartic transaminase (Nepochelovich, N. S., Burtseva, L. B.), cholinesterase (Dvoryaninovich, L. N.) and SH groups (Karput', S. N.) significant differences were found. Average data are presented in Tab. I.

Thiamine administration, therefore, affects the metabolism of the myocardium. Even if in beri-beri the metabolic disturbances have been often studied, no reports of the effects of hypervitaminosis B₁ are known to us. Particularly in the latter state we have met with increases in enzyme activities. Oxidation and anaerobic breakdown of carbohydrate is increased, protein metabolism is activated and there are changes in the rate of red.-ox. reactions of thiamine itself. In summary, these results to a certain degree explain the increased relative weight of the heart in hypervitaminosis B₁.

The state of fat metabolism was determined according to the levels of cholesterol and phospholipids (Tab. I). It is important to stress that differences from the norm in avitaminosis or hypervitaminosis are accompanied at first glance by identical changes: cholesterol content increases, phospholipids decline. The phospholipid: cholesterol ratio decreases in avitaminosis and hypervitaminosis. However, it is clear that the mechanism of the disturbance in cholesterol metabolism in these two cases is quite different. In avitaminosis there is a primary disturbance of carbohydrate metabolism, and a resultant increase in the breakdown of neutral fat to acetyl co-enzyme A. This cannot be completely utilised, and it is possible that the latter is partly used in increased cholesterol synthesis. As a result of the avitaminosis the pigeons lose almost all of their neutral fat from depots, but have raised blood and tissue cholesterol contents.

In the case of hypervitaminosis B₁ both pathways of carbohydrate breakdown are activated, resulting on the one hand in a raised amount of acetyl co-enzyme A of carbohydrate origin, and on the other hand in an excess of reduced triphosphopyridine nucleotides, at the expense of activation of the pentose phosphate cycle, and utilising the excess of co-

enzyme A for synthesis of neutral fat or cholesterol (21). The increased deposition of fat in subcutaneous tissues and about the heart in pigeon hypervitaminosis B₁ therefore has some theoretical basis. It should be stressed that the ability of excess thiamine to activate cholesterol synthesis has been previously observed on the basis of other experimental and clinical results (12, 22).

In generalising from these results, the hypothesis is put forth that administered excess thiamine actively influences the metabolism of the myocardium and can, to a certain degree, be an etiological factor in certain pathological processes, atherosclerosis in the first instance, and cardiac components of hypertension as well. On the other hand, in disease states associated with cardiac insufficiency, thiamine can play an important role in normalising cardiac function.

The experiments on the rice diet have, however, one basic disadvantage: nutritional inadequacy. For this reason complementary experiments were carried out, thus far on 22 pigeons, so that it must be considered as preliminary at present, long term administration of thiamine was carried out and the effect on myocardial metabolism was observed. The hypervitaminosis group (11 pigeons) received 1.5 to 2.0 mg. of thiamine daily per os. The parameters followed did show the same changes not in all respects as on the rice diet. The first fact to draw the attention is that control and hypervitaminosis birds showed the same heart weight. It would appear that the effect of thiamine and rice diet is based on a complementary action along with some other deficiency of this diet, so that the specific effect of thiamine on the heart is based on some other deficiency of diet. A further factor is that birds on the sufficient diet were heavier at the end of the experiments than the control birds, so that the relative weight calculation was shifted. It is possible that these differences are due to the presence in rice of antithiamine factors such as orizotoxin (23), which can *per se* play a role in the production of beri-beri.

The importance of these results is that we can consider not only hypovitaminosis pathologies of the myocardium, but hypervitaminosis pathologies as well.

SUMMARY

Ostrovskii, Y. M. (Dept. of Biochem., State Med. Inst., Grodno, USSR): *Concerning the specific sensitivity of the heart to the administration of thiamine.* Cor et Vasa 4(4):289-295, 1962.

The effect of experimental avitaminosis and hypervitaminosis B₁ on the heart and some metabolic processes in the myocardium was studied in 107 pigeons. In heart homogenates from various groups generally accepted colourimetric methods were used to estimate aldolases, glutamic-aspartic transaminase and cholinesterase. Transketolase was determined by the amount of sedoheptulose-7-phosphate formed from ribose-5-phosphate, thiamine-dehydrase according to the amount of thiochrome. Sulfhydryl groups were titrated with silver nitrate with a rotating platinum electrode.

With hypervitaminosis on a rice diet the relative weight of the heart increased and the following enzyme systems were activated: transketolase, thiamine dehydrase, aldolase, transaminase. Cholinesterase activity did not change. The amount of free sulfhydryl

groups was increased, along with cholesterol, and phospholipid concentrations were lowered.

Changes observed during avitaminosis and hypervitaminosis B₁ in myocardial metabolism demonstrate the specific sensitivity of this organ to thiamine administration.

Probably some pathological states of the myocardium can be produced not only by avitaminosis but also by hypervitaminosis.

ZUSAMMENFASSUNG

An 107 Tauben wurde der Einfluss experimenteller Avitaminose und Hypervitaminose B₁ auf das Herz und einige Stoffwechselvorgänge im Myokard untersucht. Im homogenisierten Herzmuskel der verschiedenen Taubengruppen wurde unter Anwendung allgemein anerkannter kolorimetrischer Methoden die Aktivität von Aldolase, Glutamin-Asparaginsäuretransaminase und Cholinesterase untersucht. Die Transketolase wurde aus der Menge des aus Ribose-5-Phosphat entstandenen Sedoheptulose-7-Phosphates und die Thiaminodehydrase aus der Menge des Thiochroms bestimmt. Freie Sulfhydrylgruppen wurden mit Silbernitrat unter Verwendung einer rotierenden Platinelektrode titriert.

Bei Hypervitaminose unter Reisdiät nimmt das relative Gewicht des Herzens zu und die Aktivität folgender Fermentsysteme wird erhöht: Transketolase, Thiaminodehydrase, Aldolase, Transaminase. Die Aktivität der Cholinesterase bleibt unverändert. Die Menge der freien Sulfhydrylgruppen und des Cholesterins nimmt zu, die Konzentration der Phospholipide nimmt ab.

Die bei Avitaminose und Hypervitaminose B₁ beobachteten Veränderungen im Myokardstoffwechsel sprechen für hohe Empfindlichkeit des Herzens auf Thiaminzufuhr.

Es scheint, dass einige pathologische Zustände des Herzmuskels nicht nur durch Hypo- bzw. Avitaminose, sondern auch durch Hypervitaminose B₁ hervorgerufen werden können.

RÉSUMÉ

Sur 107 pigeons, on a étudié l'influence de l'avitaminose et de l'hypervitaminose B₁ expérimentales sur le cœur et certains mécanismes métaboliques du myocarde. Dans les échantillons du muscle cardiaque homogénéisé de différents groupes des pigeons, on a évalué, par des méthodes colorimétriques universellement acceptées, l'activité de l'aldolase, de la transaminase glutamo-aspartique et de la cholinestérase. La trans-cétolase a été dosée d'après la quantité de sédoheptulose-7-phosphate, formé à partir du ribose-5-phosphate, la thiaminodéshydrase d'après la quantité du thiochrome. Les groupes sulfhydriques ont été titrés par le nitrate d'argent, à l'aide d'une électrode en platine rotatrice.

Lors de l'hypervitaminose provoquée par le régime au riz, le poids relatif du cœur augmente et l'activité des systèmes enzymatiques que voici s'intensifie: trans-cétolase, thiaminodéshydrase, aldolase, transaminase. L'activité de la cholinestérase ne change pas. Il y a augmentation des groupes sulfhydriques libres et du cholestérol, le taux des phospholipides diminue.

Les changements observés dans le métabolisme du myocarde, au cours de l'avitaminose et de l'hypervitaminose B₁ démontrent la sensibilité particulière du cœur vis-à-vis l'approvisionnement en thiamine.

Vraisemblablement, certains états pathologiques du muscle cardiaque peuvent être provoqués non seulement par l'avitaminose ou l'hypovitaminose, mais aussi par l'hypervitaminose.

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Toxic Effects of an Excess of Vitamin B₁ in Rats.

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Incidental to other studies on the effect of an excess of vitamin B₁ on resistance, certain unusual toxic manifestations of an excess were observed. We have been unable to find any report in the literature describing similar effects.

The rats used in these experiments were of Wistar stock, raised in our laboratories and free from *Bartonella muris*. Three groups of animals, 4 weeks old, were used. All 3 groups were fed a complete diet consisting of 15 gm. per rat per day of a basic mixture of hominy 100 parts, rolled oats 25 parts, fine meat and bone 25 parts, salt 1½ parts and dried skimmed milk 16 parts, to which was added a few drops of cod liver oil, 0.3 mg. of wheat germ and 0.3 gm. of crude Fleischman's brewer's yeast per rat. This yeast product contains 6 I.U. of B₁ per gram. This is equivalent to about 2 I.U. per rat per day. In addition, the animals received fresh greens twice a week and fresh milk every day. On this complete diet, it has been our experience that the rats maintain a good growth curve, reproduce well and rear all their young without loss of any members of their litters.

In the present experiment, one group containing 8 females was fed this complete diet, but supplemented with Mead Johnson's brewer's yeast in amounts equivalent to 50 I.U. of vitamin B₁ per rat per day. A second group containing 6 females was given supplements of vitamin B₁ concentrate (adsorbate, Eli Lilly) in amounts equivalent to 50 I.U. per day. The vitamin B₁ adsorbate was subsequently replaced by synthetic vitamin B₁ (Betaxin) and this was given in equivalent unitage, administered subcutaneously. A third group in which there were 9 females maintained on the standard complete diet alone, was used as controls. The experiment was continued for several generations.

Of the control group, within a period of 4 months (in the winter) 5 became pregnant and had normal litters of 7 to 10 young, all of which grew to maturity. The other 4 did not conceive in this period. The offspring continued to breed at maturity as usual. In common with the experience of others, we have observed fluctuations in the rate of reproduction dependent on season, but only rarely have we observed instances in which a litter was not normally raised.

The rate of growth of the animals fed an excess of B₁ concentrate, or synthetic vitamin B₁, was no greater than that of the group of animals fed the normal complete diet. The females of these groups bred more regularly than the controls. However, in the first generation of rats fed the excess B₁ concentrate, 2 of their 7 litters were born dead, apparently due to premature parturition; 2 were born alive, but died and were eaten within 24 hours; and 3 litters of 3 to 8 young grew to maturity. Before the second mating, the B₁ concentrate was replaced by synthetic vitamin B₁ (Betaxin) in equivalent unitage injected subcutaneously. One mother that had eaten its first litter, when given Betaxin instead of B₁ concentrate, became pregnant a second time and threw a litter of 4 healthy young that grew to maturity. The second generation on Betaxin, however, when bred, showed the same toxic effects. In 3 litters the young were born alive but the mothers neglected their young and within 48 hours had devoured most of the offspring.

The animals fed an excess of vitamin B₁ in the form of Mead Johnson's brewer's yeast, thrived much better than the controls or those fed vitamin B₁ concentrate as far as the rate of growth was concerned. Throughout the experiment, the animals weighed on an average of 50% more than did the members of the other 2 groups. (This is consistent with the findings of Williams, Waterman and Keresztesy¹ who noted improved size of rats fed increasing amounts of vitamin B₁ up to a limit of 100 times the minimal protective dose.) Seven of 8 females conceived and gave birth to litters of 6 to 11 young at a time of the year when breeding in the control stock was at a minimum. However, in 2 litters, the young were ignored by the mothers, and they died of starvation, a defect of lactation apparently being present. The other 5 litters grew to maturity, their growth curve exceeding the controls. This second generation grew well and showed no toxic effects from the excess. However, when they became mature, some were inbred with their litter mates and of the 9 litters of this second generation, in 2 the mothers showed no interest in the young and failed to lactate adequately. As a result, the young became emaciated and starved to death. In 4, the mothers ate the young within 24 hours. When it was noted that the first 6 litters born of the mothers in this second generation were lost, the excess yeast was discontinued for a period of 2 weeks and then readministered in amounts of 20 I.U. per day to the remaining pregnant females. Of the next litter born after this change in quantity

¹ Williams, R. R., Waterman, R. E., and Keresztesy, J. C., *Science*, 1935, 81, 586.

of excess yeast administration, 3 of the young survived. The remaining young of this litter were not nursed and starved to death. Litters born within a period of 4 weeks after the reduction of the excess of yeast were perfectly normal, all surviving. The mothers lactated adequately and showed the normal interest in their young. It is interesting that new-born rats of the stock given an excess of yeast for 2 generations, injected daily subcutaneously with 20 to 50 I.U. of synthetic vitamin B₁, did not show any perceptible difference in the time of weaning from those of the control normal stock fed our standard adequate diet.

Since the groups receiving excess yeast, concentrated B₁ and synthetic B₁ showed similar toxic effects, it is definite that the common responsible factor was B₁ and not other elements in the B₁ adsorbate or other factors in the yeast.

Leong² observed that the maximum storage of vitamin B₁ is attained following the administration of 30 I.U. per rat per day in a period of 4 to 6 weeks. From our own observations, it would seem that an excess of vitamin B₁ in amounts exceeding 20 or 30 I.U. a day during a period of more than one generation may interfere with lactation and the normal maternal instinct, resulting in high litter mortality and cannibalism. Nakahara, Inukai and Ugami^{3,4} have recently described an "L-factor" in liver and yeast which they state is a specific dietary factor necessary for lactation. It is not present in the concentrated vitamin B₁ adsorbate. It is possible that vitamin B₁ in great excess may inhibit the action of such a lactation factor.

Summary. Additions of Mead Johnson's brewer's yeast in amounts equivalent to 50 I.U. of vitamin B₁ per animal per day will cause a marked increase in the weight curve for several generations. This amount of yeast, however, results in a disturbance in lactation and a loss of the nursing instinct evident in the first generation, but almost universal in the second generation, with consequent starvation of the young, and a high incidence of cannibalism. The effect was more pronounced in those animals fed B₁ concentrate (adsorbate). Among these, still-births were common as well. Synthetic vitamin B₁ was somewhat less toxic in excess amounts than the vitamin B₁ adsorbate but similar interference with lactation and the nursing in-

² Peng Cheng Leong, *Biochem. J.*, 1937, 31, 367.

³ Nakahara, W., Inukai, F., and Ugami, S., Abstr. in *Ber. d. d. g. Physiol. u. Exp. Pharm.*, 1937, 93, 143; *Sol. Pap. Inst. Physic. Chem. Res.*, 1935, 28, 152.

⁴ Nakahara, W., Inukai, F., Kato, S., and Ugami, S., Abstr. in *Ber. d. d. g. Physiol. u. Exp. Pharm.*, 1937, 93, 140; *Sol. Pap. Inst. Physic. Chem. Res.*, 1935, 28, 47.

stinct in the second generation occurred. It is interesting that vitamin B₁ in amounts equivalent to 40 times the maintenance requirement seems to have a similar effect in interfering with the capacity of the mother to rear her young and the nursing instinct as does a relative insufficiency of this vitamin. This should in no way discourage the generous use of vitamin B₁ in the diet of human beings, as the difference between the optimal requirement and the amount which may prove toxic after several generations is considerable. The excess amount given to our rats would be equivalent to the administration of between 5,000 and 10,000 I.U. of vitamin B₁ per day for several generations (assuming that the vitamin B₁ requirement of humans is between 100 and 200 units per day).

I am indebted to Mead Johnson for the supply of brewer's yeast for these and other experiments, to Eli Lilly for vitamin B₁ concentrate, and to Winthrop Chemical Company for the generous supply of Betaxin (synthetic vitamin B₁).

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**Metabolic Interdependence of Vitamin B₁ and Manganese.
Reciprocal Neutralization of Their Toxic Effects.***

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We observed that rats fed our standard adequate, varied diet, supplemented with 100 gamma of vitamin B₁ daily, either in the form of yeast, or as synthetic vitamin B₁ (parenterally administered), demonstrated after one generation interference with lactation, loss of the maternal instinct, cannibalism and progressive loss of fertility.¹ With reduction in the amount of vitamin B₁ to 40 gamma or the elimination of the supplements of vitamin B₁ for short periods, normal lactation and normal interest in the young was restored. When the vitamin B₁ content was again increased the same toxic effects were observed.[†] Further study completely confirmed our earlier findings. With daily supplements of 60 gamma of vitamin B₁, progressive decrease in fertility also occurred, with a moderate incidence of loss of litters due to cannibalism. After four generations breeding decreased.

* A preliminary note of this work appeared in *Science*, 1939, **88**, 2302. Read before the American Society of Experimental Pathology, April, 1939, at Toronto.

¹ Perla, D., Proc. Soc. Exp. Biol. and Med., 1937, **37**, 169.

[†] Interference with lactation and reproduction could not have been due to the absence of vitamins L₁ and L₂ recently postulated by Nakahara Inukai and Ugami (*Science*, 1938, **87**, 372) since yeast is a rich source of these factors.

The data of these earlier preliminary experiments are given in detail in Table I.

Our normal diet consists of the following: 15 g per rat per day of a basic mixture of hominy 100 parts, rolled oats, 25 parts, fine meat and bone 25 parts, salt 1-2 parts and dried skimmed milk 16 parts, to which are added a few drops of cod liver oil, 0.3 mg wheat germ and 0.3 g of crude Fleischmann's brewer's yeast per rat. The yeast product contained 6 I.U. of vitamin B₁ per gram. This is equivalent to about 2 to 3 I.U. (7 gamma) per rat per day. In addition the animals received fresh greens twice a week and fresh whole milk daily. On this complete diet it has been our experience that the rats maintain a good growth curve, reproduce well and rear their young without loss of any members of their litters.

In view of the fact that Williams² stated that as much as from 160 to 1000 gamma of vitamin B₁ daily could be given without any toxic effects when rats were fed a Sherman breeding diet (one-third whole milk and two-thirds whole wheat), it seemed probable to us that interference with some other factor in the diet might have induced the manifestations observed in our experiments.

It is known that deficiency of manganese in the diet presents similarly toxic effects on the maternal instinct and reproduction.³ It was reasoned that perhaps manganese is essential as an oxidative catalyst in the utilization of vitamin B₁ in the tissues. If this were so the available manganese in the tissues might be exhausted by an excess of vitamin B₁ and manifestations would occur analogous to those observed in manganese deficiency. Furthermore, it was observed by Hamamoto⁴ that large amounts of both vitamin B₁ and manganese are found together in nature in such sources as wheat products and the like.[†]

To test our hypothesis we added small amounts of manganese (2 mg per rat per day as MnCl₂) to the diet. Rats which had shown loss of maternal instinct and cannibalism now bred and raised normal litters (Table I). The studies were then extended. Rats were raised on the normal diet and given parenterally 400 gamma of vitamin B₁ daily. Others were given the same diet and vitamin B₁ but the diet was supplemented with 2 mg of manganese as MnCl₂ per day per rat. The results completely confirmed our hypothesis. In those receiving the vitamin B₁ alone, cannibalism

² Williams, E. R., and Spies, T. D., *Vitamin B₁ and Its Use in Medicine*, Macmillan Co., New York City, 1938, p. 286.

³ Orent, E. R., and McCollum, E. V., *J. Biol. Chem.*, 1931, **92**, 651.

⁴ Hamamoto, E., *Orient. J. Dis. Infants*, 1935, **12**, 21, 57.

[†] He observed a decrease in manganese in the tissues of beri-beri birds.

TABLE I.
Toxic Effects of Excess of Vitamin B₁ on Maternal Instinct and Reproduction.

Group	Yeast Excess									Betaxin Excess								Controls				
	100 100 50 50 0 100 100 50 50 + MN									100 100 40 0 100 100 50 50 + MN								Normal Diet Alone (7 gamma B ₁)				
Generation	P	F ₁	F ₁	F ₂	F ₂	F ₂	F ₂	F ₂	F ₄	P	F ₁	F ₂	F ₂	F ₂	F ₂	F ₂	F ₄	P	F ₁	F ₂	F ₂	F ₄
No. females breeding	8	7	13	13	5	2	6	6	4	6	6	6	6	6	6	6	6	9	8	8	8	8
No. litters	7	6	3	3	5	2	3	1	2	7	3	2	6	3	4	3	3	17	16	18	15	19
No. offspring	54	32	21	32	48	12	19	8	15	39	21	12	43	20	20	29	29	139	128	168	118	150
No. litters abandoned or eaten	2	6	0	3	1	8	0	0	2	4	2	2	4	0	6	0	0	0	0	0	0	0
No. offspring abandoned or eaten	16	30	0	30	10	2	0	0	12	10	13	12	21	0	2	0	0	0	0	0	0	0

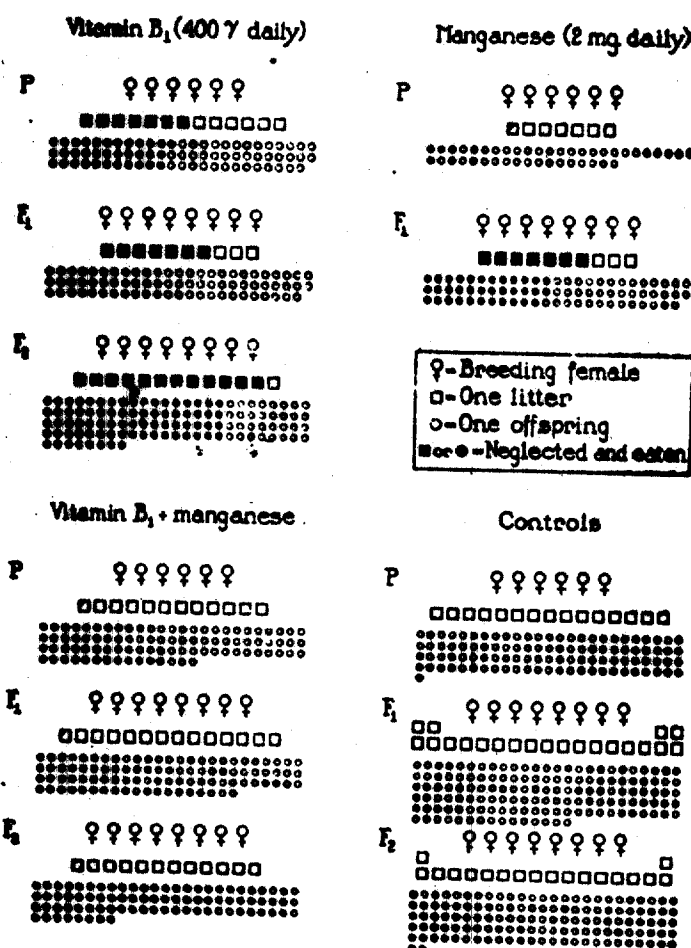
TABLE II.
The Preventive Action of Supplements of Manganese to the Diet on the Effects of an Excess of Vitamin B₁ in Rats Fed Complete Diets.*

Group	Betaxin (400 gamma daily)				Betaxin (400 gamma) + MN (2 mg daily)				MN (2 mg daily)			Controls			
	P	F ₁	F ₂	Total	P	F ₁	F ₂	Total	P	F ₁	Total	P	F ₁	F ₂	Total
Generation	P	F ₁	F ₂	Total	P	F ₁	F ₂	Total	P	F ₁	Total	P	F ₁	F ₂	Total
Total No. females breeding	6	8	8	22	6	8	8	22	6	8	14	6	8	8	22
Total No. litters	13	10	13	36	13	14	18	45	7	10	17	15	21	18	54
Total No. offspring	74	74	108	256	89	94	90	273	43	74	117	105	140	127	372
No. litters neglected or eaten	7	7	12	26	0.3	0.2	0.1	0.6	0.3	7	7.3	0	0	0	0
	54%	70%	92%	72%	2%	1%	.8%	1.3%	4%	70%	43%				
Total No. offspring neglected or eaten	34	33	76	143	3	2	1	6	2	33	35	0	0	0	0
	46%	44%	70%	56%	3%	2%	1%	2%	4%	44%	30%				

* The vitamin B₁ was given parenterally from the age of 3 weeks in amounts of 400 gamma per day but the young in each generation were not disturbed for 2 to 3 weeks. Cannibalism and deaths from neglect occurred in more than one litter in any given female in 11 instances. It frequently occurred in the 2d or 3d litter and not in the first. In only 2 instances did it occur only in a first litter. In all cases the loss of young occurred in the first 4 or 5 days.

and interference with lactation occurred in a high percentage in the P, F₁, and F₂ generation in successive litters. In those receiving supplements of manganese in the diet, none of these toxic symptoms was apparent. Normal lactation and the normal maternal instinct were preserved (Table II).

Neutralization of toxic effects of an excess of either vitamin B₁ or manganese by appropriate ratio of manganese to vitamin B₁



All rats received a complete normal diet containing a minimum of 2 to 3 I.U. of vitamin B₁ (77)

FIG. 1.
Breeding chart illustrating the neutralization of the toxic effects of an excess of either vitamin B₁ or manganese by appropriate ratio of manganese to vitamin B₁.

It is extremely significant that the animals receiving the normal diet to which was added a supplement of manganese alone in amounts of 2 mg a day, likewise showed a disturbance in lactation and in maternal instinct, which was slight in the first generation (P), but pronounced in the second generation. In spite of this fact, the rats receiving both excess of vitamin B₁ and excess of manganese (each in themselves capable of inducing these toxic effects) in practically every instance reared their young normally.

In work now in progress it has been found that manganese in amounts of about $\frac{1}{2}$ mg a day per rat is more effective in neutralizing the toxic effects of an excess of vitamin B₁ (400 gamma), and in itself is less toxic than 2 mg.

These results demonstrate that manganese is essential in the utilization of vitamin B₁ in the tissues and is intimately bound up with the rôle of vitamin B₁ in the physiology of the organism. It also suggests that variations in certain constituents of the diet, such as manganese may greatly affect the vitamin B₁ requirement. With the use of large amounts of vitamin B₁ in therapy, an adequate supply of manganese must also be made available.

Our experiments further suggest that in the presence of an excess of manganese, a greater quantity of vitamin B₁ is essential. Perhaps the vitamin B₁ in the diet is rapidly exhausted under these conditions and insufficient quantities are available for normal lactation. In any case, apparently, the toxic manifestations observed with an excess of vitamin B₁ are the expression of an exhaustion of available manganese stores in the body, and the symptoms are those of insufficiency of manganese.

Summary and Conclusions. In rats fed normal adequate diets an excess of vitamin B₁ in amounts exceeding 30 or 40 times the minimal requirement results in an interference with the capacity of the mother to rear her young and with the nursing instinct. With an excess of 400 gamma this manifestation was pronounced in the parent generation but became progressively worse in the F₁ and F₂ generation. The young were neglected and eaten in over 90% of the litters in the F₂ generation.

The toxic manifestations of an excess of vitamin B₁ were found to be dependent on the ratio of manganese to vitamin B₁ in the diet. The addition of manganese to the diet in amounts of 2 mg per rat per day completely neutralized the unfavorable effects of the excess of vitamin B₁ (400 gamma daily). Practically no interference with lactation or rearing of the young was observed in animals which received both the excess of vitamin B₁ and manganese as ad-

dition to the normal diet during 3 successive generations. Apparently manganese in amounts of 1/2 mg is even more effective.

Supplements of manganese alone in amounts of 2 mg a day result in interference with lactation and cannibalism, particularly marked after one generation.

It is inferred that manganese acts as an essential catalyst in oxidative processes in which vitamin B₁ is concerned. The vitamin B₁ requirement of an animal varies with the manganese content in its diet.

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phosphate (formed from fructose-1,6-diphosphate by aldolase) as acceptor aldehyde, the decarboxylation of hydroxypyruvate led to the formation of ribulose-5-phosphate.¹ The pentose phosphate was isolated as an alcohol insoluble barium salt and determined by two independent tests as shown in Table I. Similar results were obtained when D-glyceraldehyde-3-phosphate (Concord Laboratories) was used instead of fructose-1,6-diphosphate and aldolase.

TABLE I

ENZYMATIC FORMATION OF RIBULOSE-5-PHOSPHATE FROM
HYDROXYPYRUVATE AND TRIOSE PHOSPHATE

0.5 mg. of purified yeast transketolase (22,000 units per mg. protein) was used in these experiments. Carbon dioxide was measured manometrically. In Expt. 1, the reaction mixture (2 ml.) contained 100 micromoles of potassium phosphate (pH 6.5), 5 micromoles of fructose-1,6-diphosphate, 20 micrograms of aldolase, 12 micromoles of MgCl₂, 20 micrograms of ThPP and about 30 micromoles of sodium hydroxypyruvate. In Expt. 2, 100 micromoles of tris-(hydroxymethyl)-aminomethane (pH 6.9) was used instead of potassium phosphate and the concentration of fructose-1,6-diphosphate was increased to 10 micromoles. The vessels were incubated at 37° for 75 minutes in Expt. 1 and 175 minutes in Expt. 2. Deproteinization with 5% trichloroacetic acid was followed by the isolation of an alcohol-insoluble barium salt which was analyzed colorimetrically as well as spectrophotometrically. In the latter test transketolase free of pentose isomerase was used and triose phosphate formation was measured with either glyceraldehyde, glyceraldehyde, or ribose-5-phosphate as "acceptor aldehydes."

Expt.	CO ₂ liberation, micromoles	Isolated ribulose-5-phosphate, micromoles Orcinol reaction	Spectrophotometric
1	4.9	1.8	1.6
2	4.0	3.1	2.9

TABLE II

THIAMINE PYROPHOSPHATE REQUIREMENT OF TRANSKETOLASE

The enzyme preparation was dialyzed against 1000 volumes of 0.6% Versene in 0.02 M potassium phosphate of pH 7.4 for 20 hours and then against 1000 volumes of 0.6% Versene in 0.9% KCl for another 20 hours. The enzyme was assayed by measuring triose phosphate formation from ribulose-5-phosphate in the presence of ribose-5-phosphate as "acceptor aldehyde."

Enzyme preparation	Additions to test system	Activity (units per ml.)
Undialyzed	50,000
Dialyzed for 40 hours	2,000
	3 μ M. MgCl ₂	5,000
	50 μ g. ThPP and 3 μ M. MgCl ₂	43,000
Dialyzed for 40 hours then left in icebox for 24 hours	500
	3 μ M. MgCl ₂	1,000
	50 μ g. of ThPP	7,000
	50 μ g. of ThPP and 3 μ M. of MgCl ₂	40,000

Since the formation of ribulose-5-phosphate represents a ketol condensation, and no free glycolaldehyde is formed, one must assume the formation of an "active glycolaldehyde" which condenses with the "acceptor aldehyde" to form a ketosugar. The enzyme may therefore be termed a transketolase.

THIAMINE PYROPHOSPHATE, A COENZYME OF
TRANSKETOLASE

Sir:

In a note by Horecker and Smyrniotis¹ previous work on enzymes concerned in the breakdown of pentose phosphate is quoted. We have isolated from baker's yeast a crystalline enzyme which catalyzes the cleavage of ribulose-5-phosphate with the formation of D-glyceraldehyde-3-phosphate, identified by means of glyceraldehyde-3-phosphate dehydrogenase free of triose isomerase. The cleavage of ribulose-5-phosphate² occurs only on the addition of an "acceptor aldehyde" such as ribose-5-phosphate, glycolaldehyde, or glyceraldehyde. The enzyme was also found to decarboxylate hydroxypyruvate in the presence of an "acceptor aldehyde." With D-glyceraldehyde-3-

(1) B. L. Horecker and P. Z. Smyrniotis, THIS JOURNAL, 75, 1009 (1953).

(2) We wish to thank Dr. B. L. Horecker for a gift of ribulose-5-phosphate.

(3) A similar reaction catalyzed by rabbit muscle mince has been described by S. Akaburi, Kibachiro Uehara and I. Muramatsu, *Proc. Japan Academy*, 28, 39 (1952).

The activity of a partially purified transketolase from *E. coli* was doubled by the addition of thiamine pyrophosphate (ThPP). No requirement for ThPP was found with a twice recrystallized preparation of the yeast enzyme, but extensive dialysis against a Versene-KCl solution caused nearly complete inactivation. Addition of magnesium chloride and of ThPP to the dialyzed enzyme restored the activity, as shown in Table II. The crystalline yeast enzyme shows no aldolase, triose isomerase or pentose isomerase activity.

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**PRELIMINARY STUDIES ON STABILIZATION OF THIAMIN
IN DEHYDRATED FOODS¹**

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Dehydrated and canned products of all types frequently are subjected to unfavorable storage owing partly to unavoidable shipping and warehouse conditions and partly to the assumption that such foodstuffs are entirely stable. The fact that foods of these types can survive long periods under adverse conditions without definite spoilage contributes to the erroneous belief that they are nutritionally stable. Unfortunately neither dehydrated nor canned foods retain their nutritive value completely under all conditions. In some products there are serious losses of vitamins, especially thiamin, during storage unless certain precautions are observed. The general acceptance of pork products as excellent sources of thiamin emphasizes the importance of this problem in dealing with dehydrated meats.

Usually losses of thiamin during storage are negligible at low temperatures, but above ordinary room temperatures losses in either dehydrated or canned products often become great. It has been reported previously that the thiamin content of dehydrated pork decreases rapidly during storage at temperatures above 37°C. (98.6°F.)—Rice and Robinson (1944a). This is not true of dehydrated pork alone since dehydrated eggs, Klose, Jones, and Fevold (1943); canned meats, Rice and Robinson (1944b); canned green beans, Farrel and Fellers (1942); and condensed milk, Knott (1942) also lose thiamin at appreciable rates while stored at temperatures ranging from 25 to 49°C. (77 to 120.2°F.).

On the other hand, skim-milk powder, wheat, soya flour, dehydrated vegetables, and certain food mixtures appear to be much more stable (Table 2). Inasmuch as some foods seem to have their thiamin "stabilized" to a significant degree, it seemed possible that they might exert a "protective" action upon the thiamin in the less stable foods if mixed with the latter. Such an effect seems to be responsible for the greater stability of thiamin in certain meat-cereal mixtures—Rice, Beuk, and Robinson (1943).

Whether a chemical substance is responsible for the stabilizing action or whether the effect is due to a physical influence cannot be ascertained from existing information. The studies reported here were conducted with the hope that the factors affecting thiamin retention might be defined more clearly.

¹ Presented before the Division of Biological Chemistry, American Chemical Society, at Pittsburgh, September, 1943.

EXPERIMENTAL PROCEDURE

General Procedures: A modification of Hennessy and Cerecedo's thiochrome procedure was used for determining thiamin—Hennessy and Cerecedo (1943). Measurements of thiochrome intensities were made with a Coleman Model 11 Photofluorometer.

Since it had been suggested that thiochrome procedures do not determine all of the thiamin in dehydrated food samples—Lane, Johnson, and Williams (1942)—it seemed advisable to check this method against biological assays. As is shown later, the two methods agreed within the limits of experimental error.

Each food item used in the stability tests was finely ground and thoroughly mixed. Control samples were stored at $-29^{\circ}\text{C}.$ ($-20.2^{\circ}\text{F}.$) for the same period of time as the test samples stored at higher temperatures. Control and test samples were analyzed simultaneously, thus avoiding errors owing to variations in standard, instrument, or technique. Retentions in the test samples were expressed as a percentage of the thiamin in the control samples, on a solids basis.

TABLE 1
Loss of Thiamin in Dehydrated Pork at Various Temperatures

Storage temperature °C.	Initial value µg./gm.	Thiamin retained		
		7 days	14 days	21 days
—29	14.5	pct.	pct.	pct.
3	14.5	100	100	100
27	14.5	100	100	96
37	14.5	89	77
49	14.5	70	55	43
63	14.5	15	7	0
		4	0	0

After initial experiments had indicated rates of loss at different temperatures (Table 1), test samples were held at $49^{\circ}\text{C}.$ in a constant temperature oven. This very severe treatment was adopted in order to obtain high losses in a short period of time. It is far above the usual temperatures encountered in handling foods and hence exaggerates the actual losses in commercial products.

Rate of Loss of Thiamin in Dehydrated Pork Stored at Various Temperatures: Exploratory experiments showed that thiamin is readily destroyed in dehydrated pork held at $37^{\circ}\text{C}.$ Therefore, it seemed desirable to determine the rates at which losses would occur at other temperatures. A single, well-mixed lot of dehydrated pork was vacuum-packed in one-pound cans and stored at the temperatures and for the intervals indicated (Table 1).

From the results it is obvious that temperatures above $27^{\circ}\text{C}.$ ($80.6^{\circ}\text{F}.$) promote rapid loss of thiamin and that storage for any reasonable length of time under such conditions will result in the destruction of most of this vitamin.

Retention of Thiamin in Various Dehydrated Products: In order to provide comparative data, a number of other foods were packaged in

cork-stoppered test tubes and stored along with the dehydrated pork samples. Since these other foodstuffs are not customarily vacuum-packed, it was considered unnecessary to so pack them in this experiment which was intended to determine losses under conditions comparable to commercial practice.

Although the moisture content of these samples varied somewhat, moisture does not seem to be correlated directly with thiamin stability. Dehydrated pork and wheat (each containing eight to 10 per cent water) showed marked differences in thiamin retention despite similar moisture levels. The same is true of dehydrated eggs and skim-milk powder, both of which contained four to five per cent moisture. Nevertheless, in subsequent experiments all samples were adjusted to six per cent moisture in order to eliminate this variable.

TABLE 2
Retention of Thiamin in Dried Products Stored 31 Days at 49°C. (120.3°F.)

Product	Thiamin
	per cent
Dehydrated skim milk.....	100
Dehydrated meat-cereal mixture ¹	94
Ground whole wheat.....	85
Dehydrated eggs.....	35
Dehydrated pork.....	10

¹ Cracked wheat, 34.2 per cent; ground barley, 21.7 per cent; soya flour, 27.6 per cent; bone meal, 7.3 per cent; dried skim milk, 5.5 per cent; salts, 2.3 per cent; tomato paste, 1.65 per cent; sardine oil, 0.25 per cent; and gum guaiac, 0.16 per cent.

The stabilities of these products differ greatly, as may be noted from the data obtained (Table 2). The excellent retention of thiamin in a dehydrated meat-cereal mixture—Rice, Beuk, and Robinson (1943)—containing 67 per cent of meat products is in sharp contrast to the great loss in dehydrated pork and illustrates the differences in stability that may be encountered in products more or less similar in composition.

Effectiveness of Various Substances in Stabilizing Thiamin in Dehydrated Pork: Since the dehydrated meat-cereal mixture lost thiamin slowly regardless of the fact that it contained approximately 67 per cent of meat, it is apparent that the non-meat portion² must be responsible for the stability. To determine if this cereal mixture would have the same effect on other meats, especially pork, all of the non-meat ingredients were combined with lean, ground pork in the proportions indicated (Table 3). In one sample sodium nitrite was omitted. These preparations were then cooked and dehydrated to six per cent moisture under identical conditions.³ Portions of the same ground pork used in preparing the pork-cereal mixture were dehydrated alone and in combination with various individual

² Cracked wheat, 34.2 per cent; ground barley, 21.7 per cent; soya flour, 27.6 per cent; bone meal, 7.3 per cent; dried skim milk, 5.5 per cent; salts, 2.3 per cent; tomato paste, 1.65 per cent; sardine oil, 0.25 per cent; and gum guaiac, 0.16 per cent. For convenience, this mixture will be referred to as "cereal mixture," although it contains non-cereal materials.

³ All products were cooked for 15 minutes at 10 pounds pressure and at 116°C. (240.6°F.).

non-meat ingredients of the cereal mixture. Samples of the various preparations were held at 49°C. for seven days for comparison with controls stored at -29°C.

There was significant improvement in thiamin retention when 33 per cent of the cereal mixture was dehydrated with the pork. When smaller amounts of this mixture or of the individual constituents were used, there was much less stabilization. The omission of sodium nitrite from the cereal mixture did not reduce its effectiveness. Because of the significance of these experiments they were repeated with similar results.

TABLE 3
Effect of Different Amounts and Ingredients of Cereal Mixture on Stability of Thiamin in Dehydrated Pork¹

Added amount or ingredient	Thiamin retained 7 days at 49°C. (120.2°F.)	
	Run 1	Run 2
	pct.	pct.
None.....	15	10
Cereal mixture, 33%.....	96	75
Cereal mixture, 22%.....	22	34
Cereal mixture, 11%.....	11	32
Cereal mixture without NaNO ₂ , 33%.....	100
Wheat, 11.5%.....	37
Barley, 7.5%.....	39
Soybean flour, 9.6%.....	33	20
Tomato paste, 1%.....	37	28
Tomato paste, 5%.....	16
Soybean flour, 20% + tomato paste, 2%.....	35

¹ All samples contained six per cent moisture.

From these data it is apparent that the cereal mixture exerted a stabilizing effect upon the thiamin in the mixed product. Dehydrated pork alone retains 10 to 15 per cent of its original thiamin for seven days at 49°C. The same pork plus 33 per cent of the cereal mixture retains 75 to 97 per cent, six times as much as the pork alone. Numerous other comparisons not recorded here substantiate these ratios.

That the effect noted is not associated specifically with any one of the major ingredients is indicated by their failure to stabilize thiamin in pork when dehydrated with it in approximately the same proportions as when included in the cereal mixture. Thus, meat dehydrated with 11.5 per cent wheat failed to show much stabilization. Similarly, no other individual ingredient improved retention markedly when present in the same concentration as in the cereal mixture. However, when 33 per cent of wheat, barley, soybean flour, or dried skim milk were cooked and dehydrated with pork, the stabilization was as pronounced as that resulting from the use of a similar quantity of the entire cereal mixture (Table 4). It thus appears that a number of materials are capable of stabilizing thiamin if included in sufficient quantities.

The stabilization affects the thiamin in the meat as well as that in the cereal, since retention in a meat-cereal mixture is better than the sum of the individual retentions. One hundred grams of the pork-wheat mixture

referred to (Table 4) contained 1.13 mg. of thiamin. Of this, pork contributed 1.04 mg. while wheat supplied only 0.09 mg. If the wheat and pork had been stored separately, retentions at the end of the storage period would have been 0.21 mg. (1.04×20 per cent) in the pork and 0.08 mg. (0.09×85 per cent) (Table 2) in the wheat, or a total of 0.29 mg. The cooked and dehydrated mixture, however, retained 0.99 mg. (1.13×85 per cent) or more than three times as much as the sum of individual retentions. Thus, the wheat obviously influences the rate of thiamin destruction in the meat.

TABLE 4
Effect of Ingredients of Cereal Mixture on Stability of Thiamin in Dehydrated Pork

Addition	Thiamin retained 7 days at 49°C. (120.2°F.)	pH
	pct.	
None.....	20	6.30
Cereal mixture, 33%.....	89	6.25
Wheat, 33%.....	88	6.15
Barley, 33%.....	88	6.08
Soybean flour, 33%.....	75	6.38
Soybean flour, wheat, and milk, 11% each.....	88	6.29
Dried skim milk, 33%.....	88	6.30
Bone meal, 5%.....	23

Since samples showing a wide range of stability had similar pH values (Table 4), there is no indication that differences in acidity are responsible for the various rates of thiamin loss.

Various substances known to stabilize other constituents of foods were not very effective in stabilizing thiamin in dehydrated pork. In addition to the substances listed (Table 5), lecithin and thiourea were also ineffective.

TABLE 5
Effect of Various Substances in Stabilizing Thiamin in Dehydrated Pork

Substance added	Thiamin retained 7 days at 49°C. (120.2°F.)
	pct.
None.....	11
Nipa, 0.1%.....	20
NaCl, 5%.....	3
Gum guaiac, 1%.....	15
Avonox, 2%.....	27
Avonox, 5%.....	24
Avonox, 10%.....	24
Glucose, 5%.....	25
Dried milk, 5%.....	20

Influence of Atmosphere Within Package on Thiamin Stability: That atmospheric oxygen is not responsible for the rapid loss of thiamin from pork was demonstrated by determining the thiamin content of replicate samples after storage at 49°C. for one week in vacuum, air, nitrogen, or

carbon dioxide (Table 6). In fact, the retention of thiamin was slightly greater in the samples stored in air at 49°C. than under the other conditions. Even though there may be slightly improved retention of thiamin by storage in air, the development of rancidity makes such a procedure impractical.

TABLE 6
Effect of Storage Conditions on Thiamin Retention

Storage condition	Thiamin retained 7 days at 49°C. (120.2°F.)	
	Run 1	Run 2
Packed in vacuum.....	pet.	pet.
Packed tightly in air.....	2	0
Packed loosely in air.....	15	14
Packed loosely in CO ₂	28
Packed loosely in N ₂	11
	0

Influence of Moisture on Thiamin Stability: Although moisture is not the sole factor determining the stability of thiamin, as pointed out above, under certain conditions it greatly influences the rate of loss. To determine the extent of its influence, dehydrated pork was held in a vacuum over phosphorus pentoxide until its weight became constant. Samples of various moisture levels were then prepared and stored at 49°C. for a week. Anhydrous pork retained its thiamin well; but as the moisture content increased, the rate of loss also increased until at six per cent it had reached a maximum (Table 7). This makes it seem possible that moisture is one of the factors involved. On the other hand, canned pork with a moisture content of 50 to 55 per cent is much more stable than dehydrated pork, losing only 15 to 18 per cent of its thiamin in seven days at 49°C., Rice and Robinson (1944b), a striking contrast to the 85 to 90 per cent loss ordinarily shown in dehydrated pork. It is obvious, therefore, that moisture content *per se* is not the sole factor involved in thiamin breakdown, although complete dehydration is one means of lessening thiamin loss. From a commercial standpoint the production of anhydrous pork is impractical.

Biological Analyses: Male Sprague-Dawley rats initially weighing 35 to 45 grams were fed a thiamin-free diet⁴ until they showed growth failure and polyneuritic symptoms (20 to 25 days). The rats were then distributed into six groups in accordance with the principles prescribed by the U. S. Pharmacopoeia XII, each group consisting of 10 individually caged rats. In addition to the basal thiamin-free diet (fed *ad libitum*) daily supplements as indicated (Table 8) were administered for 28 days. These supplements were readily consumed when mixed with small portions of

⁴ The thiamin-free diet was prepared by treating 10-pound quantities of the "meat-cereal mixture" (which has been used in this laboratory with excellent success in raising rats) with 167 grams of Na₂SO₄ dissolved in a liter of water, acidifying with HCl to pH 5.5, and then autoclaving for one hour at 15 pounds pressure. The sulfite-treated material was then dried in a cabinet drier with air at 63°C. (145.4°F.). Thiochrome analyses indicated the product to be free of thiamin.

the basal diet and placed on top of the regular feed. The quantities of meat to be fed daily to provide each rat with four micrograms of thiamin were calculated from thiochrome assays which indicated the fresh pork to contain 9.6 micrograms of thiamin per gram and the dehydrated pork No. 184 and No. 184S to contain 14.5 and 1.0 micrograms per gram, respectively.

TABLE 7
Influence of Moisture Content on Stability of Thiamin

Moisture ¹	Thiamin retained 7 days at 49°C. (120.2°F.)
pct.	pct.
0	91
2	60
4	23
6	9
9	11

¹ All samples sealed *in vacuo*.

Pork No. 184S was a portion of No. 184 which had been held for two weeks at 49°C. Since direct biological analysis of No. 184S would have necessitated the feeding of large supplements, it was fed at the same level as No. 184 with sufficient pure thiamin added to make the thiamin intake four micrograms per day, assuming the thiochrome values to be correct. While this procedure cannot be expected to yield an exact analysis of the product, it is a sensitive way of detecting "bound" thiamin.

All groups receiving the calculated four micrograms of thiamin in fresh or dehydrated pork grew at a rate equal to the group fed four micrograms of pure thiamin (Table 8). Therefore, within experimental error, the two methods check. The failure of the group receiving the stored dehydrated pork plus pure thiamin to grow more rapidly than the control group proves that the low thiamin values found for such samples by thiochrome analyses are not more than a few per cent in error.

DISCUSSION

Although definite reduction in the loss of thiamin in dehydrated pork may be achieved by the use of high levels of cereals or dried skim milk, the mechanisms involved are rather obscure. Since thiamin is quite readily inactivated by hydrolytic cleavage, it might be assumed that water is responsible for its destruction in foods. The experiment showing the influence of reducing the water content of dehydrated pork from six per cent to 0 supports such a belief, but the great stability of thiamin in other foods containing as much as 50 per cent water shows that water content *per se* does not determine the degree of destruction of thiamin. However, certain substances, such as the carbohydrates or proteins which are present in cereals and dried milk, may have chemical or physical properties which enable them to inhibit the action of water on thiamin. The predominance of carbohydrate material in the stabilizing substances so far studied indicates that the factor may be associated in some manner with carbohydrate. This is made to seem even more probable since a recent experiment has proved cornstarch to be as effective as the cereals in stabilizing thiamin.

Work now in progress should determine whether or not such a conclusion is justified.

It seems rather improbable that the loss of thiamin from dehydrated pork stored at elevated temperatures is caused by enzymic hydrolysis of the nature postulated by Sealock, Livermore, and Evans (1943), since pork is cooked prior to dehydration; and these workers and others—Green, Carlson, and Evans (1941) and Woolley (1941)—studying the same phenomenon, i.e., the inactivation of thiamin by fresh-fish tissues, have demonstrated the heat lability of the thiamin-destroying factor in their preparations. It is possible, however, that even after cooking, traces of the original enzymic activity remain and that this is responsible for the effects here reported. In such cases, the excess of carbohydrate or some substance occurring with it might inhibit the reaction between thiamin and the enzyme.

TABLE 8
Biological Analyses of Pork Products

Group No.	Daily supplement	Average gain in 38 days	Thiamin intake, calculated from gain ²
1.....	None	gm. 11.6 ¹	mg.
2.....	6 micrograms crystalline thiamin	76.6	6.00
3.....	4 micrograms crystalline thiamin	60.5	4.00
4.....	0.414 gm. fresh pork containing 4 micrograms thiamin ¹	59.5	3.86
5.....	0.276 gm. dehydrated pork, No. 184, containing 4 micrograms thiamin ¹	63.9	4.41
6.....	0.276 gm. dehydrated pork, No. 184S, containing 0.28 micrograms thiamin ¹ plus 3.72 micrograms pure thiamin	61.6	4.13

¹ Five survivors. ² Measured by thiochrome method.

SUMMARY

It has been shown that dehydrated pork and dehydrated eggs stored at or above temperatures of 37°C. (98.6°F.) lose thiamin more rapidly than certain other foods, such as dried skim milk or cereals.

Dehydrated mixtures of 67 per cent pork and 33 per cent cereals (wheat, barley, or soya flour) or dehydrated milk lose thiamin much less rapidly than dehydrated pork alone.

The rate of loss of thiamin from dehydrated pork is roughly proportional to the moisture content of the sample between 0 and six per cent moisture levels. Canned pork, with moisture of 50 to 55 per cent, shows

less loss than dehydrated pork with only two per cent moisture, so factors other than this are involved in thiamin stability.

Storage of dehydrated pork in vacuum, nitrogen, or carbon dioxide rather than in air did not increase thiamin retention.

In levels as great as or greater than ordinarily used in foods, Avenex, Nipa, lecithin, glucose, and sodium chloride were ineffective in reducing thiamin loss.

Products showing greatly different stabilities may have similar pH values.

Products rich in carbohydrate materials are the most effective stabilizing agents so far studied.

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IMBALANCE OF VITAMIN B FACTORS

PYRIDOXINE DEFICIENCY CAUSED BY ADDITIONS OF ANEURIN AND CHALK

BY

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In the clinical literature during recent years there have been numerous indications of a certain interrelationship or antagonism between various factors of the vitamin B complex. Pellagrins, after treatment with nicotinic acid, often show signs of beriberi or of ariboflavinosis (Spies, Vilter, and Ashe, 1939; Sebrell and Butler, 1938, 1939; Sydenstricker *et al.*, 1940). There are recorded instances also of converse antagonisms. Thus Bichel and Moulengracht (1941) report a case of pellagra arising after treatment of the Plummer-Vinson syndrome with riboflavin (i.e., vitamin B₂); and Salvesen (1940), Brøndstrup (1940), and Lehmann and Nielsen (1939) all found signs of pellagra in patients who had been given large injections of vitamin B₁. There is little doubt that in many of these cases the deficiencies in the diet were multiple, and it is assumed that successful treatment of the predominant deficiency with a single factor of the B complex showed up other deficiencies which had previously been masked. At the same time there is the possibility, in some cases at least, that secondary deficiencies were induced by the excessive dosing with one particular factor of the B complex, and that the unbalanced proportions of the different factors gave rise to a deficiency which was not present under the original conditions. Spies, Vilter, and Ashe (1939) made the significant remark, without apparently realizing its possible implications, that in pellagrins treated with nicotinic acid and continuing on their usual diets, *the associated deficiency* (i.e., of B₁ and riboflavin) *often became worse*.

One or two attempts have been made, but without much success, to induce symptoms of deficiency of one B factor by excessive dosing with another. Klopp, Abels, and Rhoads (1943) found a transitory increase in urinary excretion of riboflavin in some patients after administration of thiamine (i.e., B₁), but could not induce either clinical or chemical evidence of riboflavin deficiency in these individuals even when large amounts of thiamine were given daily for 73 days. Unna and Clark (1942) could find no evidence of adverse effects on the growth of rats following administration of excess of individual vitamins in the presence of deficiencies of other vitamins of the B complex.

Effect of Overloading with B₁

Recent experiments on rats in this Institute, however, have produced clear-cut evidence of the adverse effects that may be caused by a disturbance of the balance of the vitamin B factors in the diet, and have shown that overloading with one component, B₁, can produce a definite deficiency of another component, B₂. The experiments were designed to follow up a series of which the results were reported a year ago. In studies of the influence of the dietary factor on reproduction and lactation the results showed the beneficial effects on the breeding performance of rats of additions of chalk, dried yeast, and milk to a poor human diet (Richards, 1943). On the other hand, addition of pure vitamin B₁, as aneurin, seemed to have a definitely harmful effect; as the weaning weights of the young rats were low and the mothers were in very poor condition after rearing their litters. It was therefore suggested that

caution must be exercised in the addition of vitamin B₁ to a poor diet, and the present experiments were planned to investigate this point more closely. Using a synthetic diet* as basal ration in place of the human dietary of the earlier experiments, vitamin B₁ (as aneurin) was given at 3 levels—low, medium, and high—and these 3 levels were repeated in 3 additional groups which received also a supplement of chalk.

In the growth tests, which lasted from weaning till the rats were mated at about 115 days of age, the chalk group at each level of B₁ intake showed a higher weight increase than the corresponding group without chalk, but these differences were not great. The groups receiving high B₁, both with and without added chalk, were somewhat lower in weight than the corresponding groups on medium B₁, but there was no noticeable difference between the animals in their general condition. Thus the growth tests, like those of Unna and Clark (1942), gave no very marked evidence of untoward effects arising from the variations in the diet. It is generally recognized, however, that a diet which may be reasonably adequate for growth, and even for reproduction, may not be adequate for successful lactation, and the lactation test in these experiments, even when the diet was improved by milk supplements, revealed differences between the groups which had been quite unsuspected from the growth test and the reproduction records. In certain cases the litters failed completely to survive to weaning; in others a few members of the litter survived, but were much below normal weight and in very poor condition; while in some instances litters which were nearly normal in weight and apparently quite healthy suddenly showed the convulsive fits which enabled their condition to be diagnosed as pyridoxine (i.e., B₆) deficiency. Chick, El Sadr, and Worden (1940)

* The synthetic diet was planned to be approximately equal in caloric value and Ca content to the original poor human diet, which contained a large proportion of white bread. It consisted of: white flour (untreated with chalk or aneurin) 1,070 g., commercial casein 400 g., dried brewers' yeast 32 g., salt mixture (McCormick 185) 33.3 g., margarine 180 g., radiostoleum (containing 1 g. α-tocopherol acetate in 50 c.c.m.) 2 c.c.m., KI 0.00616 g., and MnSO₄ 4H₂O 0.0516 g. In the groups which received extra calcium, chalk was added in the proportion officially recommended in making "enriched" white bread. A small amount of aneurin was added to the basal diet to make the B₁ content equal to that of the original basal group in the human dietary experiments. In the groups with "medium" aneurin, the amount added was equivalent to the difference in B₁ content between white and national wholemeal flour, and to the "high" aneurin groups 10 times this amount of B₁ was given. There were thus 6 groups in the experiment:

I. Basal + low B₁ II. Basal + medium B₁ III. Basal + high B₁
IV. As I + chalk V. As II + chalk VI. As III + chalk

The Ca intake for Groups I, II, and III worked out at approximately 0.33 g. per 1,000 Cal., and for Groups IV, V, and VI at 0.81 g. per 1,000 Cal. The B₁ intake for Groups I and IV was approximately 198 i.u., for Groups II and V 534 i.u., and for Groups III and VI 3756 i.u. per 1,000 Cal. The B₂, KI, and Mn supplements were added as dilute solutions daily to the liquid food in making up the ration, which was fed as a solid paste. In the breeding tests, milk supplements were added to the diet—10 and 20 c.c.m. per head respectively to half of each group in the first test, and 20 c.c.m. per head in the second test.

reported the occurrence of fits of an epileptiform nature in rats maintained for long periods on a diet deficient in vitamin B₆. The seizures were characterized by hyperexcitability and circular running, convulsive spasms, and a comatose recovery period. Still later reports, from our point of view,

accompanied by loud cries and convulsive seizures," and symptoms could be prevented or cured by pyridoxine supplements. Patton, Karn, and Longenecker (1944), who study the incidence of sound-induced seizures, also recently report the occurrence of spontaneous convulsions in young rats suck-

by mothers maintained from parturition on pyridoxine-free diets.

These descriptions fitted exactly the symptoms observed in our young rats that there seemed to be little doubt that we were dealing with a conditioned pyridoxine deficiency, since the only variables in the diet were vitamin B₆ and chalk. Comparison of the groups showed that the lactation performance deteriorated as the level of B₆ increased, and that conditions were made worse by the addition of chalk. At each level of B₆, the group receiving chalk was worse than the corresponding group without chalk. Thus the best group was Group I—the basal group with low B₆ and no added chalk. The litters were approximately normal in their weight curves, whether the milk supplement was 10 or 20 c.c.m. and convulsions were not observed in any of the litters. The worst group was Group VI, with extra chalk and high B₆. In this group 10 c.c.m. milk was quite inadequate to ensure successful lactation, only one litter surviving to weaning stage. In the section of this group receiving 20 c.c.m. milk, 5 litters reached weaning stage, with approximately average weaning weight, but 6 of the 7 litters in the group showed the typical convulsions. In Group V, which had extra chalk with medium B₆, 10 c.c.m. of milk again proved insufficient, although the performance was better than in the high B₆+chalk group with 10 c.c.m. milk. Representatives of 6 litters reached weaning stage, but there were many deaths during lactation and the few survivors were low in weight and in poor condition. Convulsions were not observed in rats which were very puny and sick. With 20 c.c.m. of milk the lactation performance was again much improved. Deaths during lactation were reduced to zero, and weaning weights were approximately normal, but the characteristic fits were observed in 3 litters. Group III, with high B₆ and no added chalk, was about equal in performance to Group V, with medium B₆+chalk, and much better than the group with high B₆+chalk. One instance of convulsions was observed in Group II, with medium B₆ and no chalk, and two instances in Group IV, with low B₆+chalk. The lactation performances of 4 of the groups are summarized in Chart I, which shows the average weight curves of the individual litters from birth, and indicates the occurrence of convulsions. Groups II and IV are not included, as the weight curves showed little departure from normal.

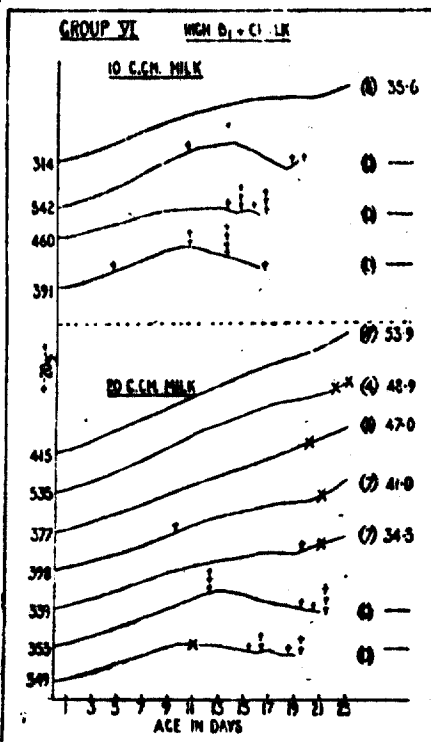
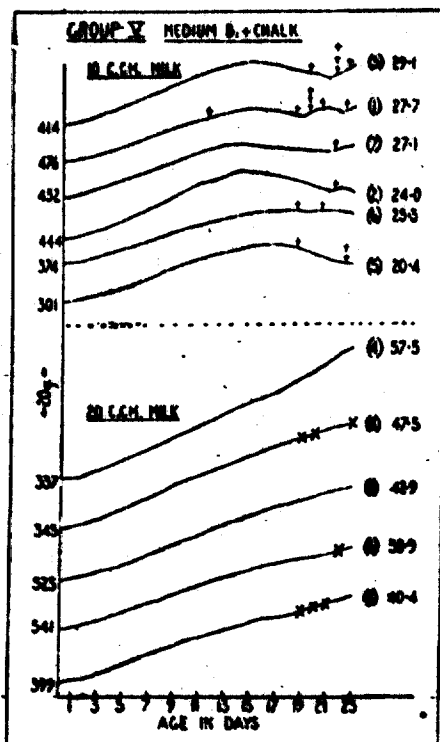
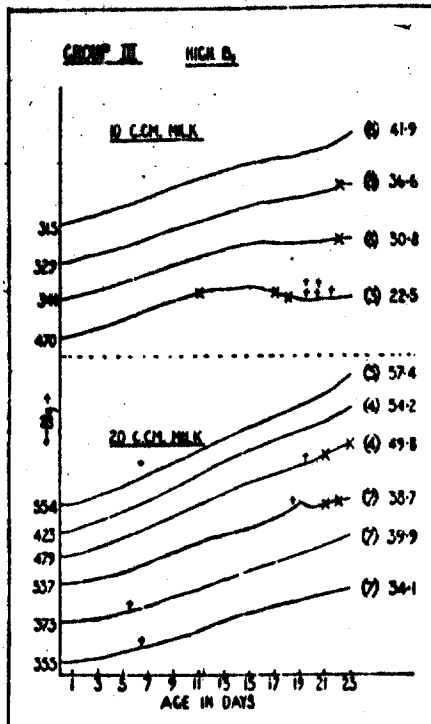
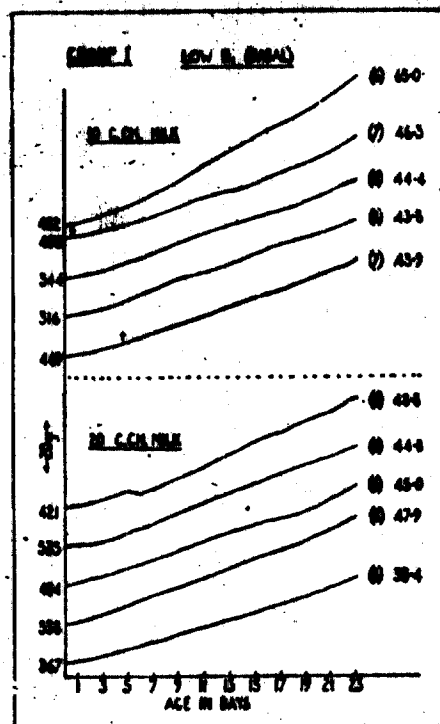


CHART I.—Showing average weight in grammes per rat from birth to weaning for individual litters. At the left of each graph is the number of the doe. At the right of each graph, in brackets, is the number of young reared. At the right of each graph, unbracketed, is the average weaning weight of the litter. † indicates the death of a rat. X indicates the occurrence of fits.

were the findings of Daniel, Kline, and Tolle (1942), who reported similar seizures in young rats while being nursed by mothers on pyridoxine-deficient diets, the symptoms appearing suddenly towards the end of the lactation period. The syndrome was "characterized by frantic running about the cage,

performances of 4 of the groups are summarized in Chart I, which shows the average weight curves of the individual litters from birth, and indicates the occurrence of convulsions. Groups II and IV are not included, as the weight curves showed little departure from normal.

The breeding test was repeated, to obtain confirmation of these results and to test whether the onset of convulsions in the young group could be prevented by giving the does a supplement of pyridoxine from parturition. This supplement was given to all the does in Groups III, V, and VI. All rats in this experiment were supplied with 20 c.cm. milk, since the 10-c.cm. supplement had proved insufficient in certain groups to bring the young to weaning and convulsions seemed to occur more readily when the young rats were fairly normal in weight. Chart II gives the results for Group VI, receiving

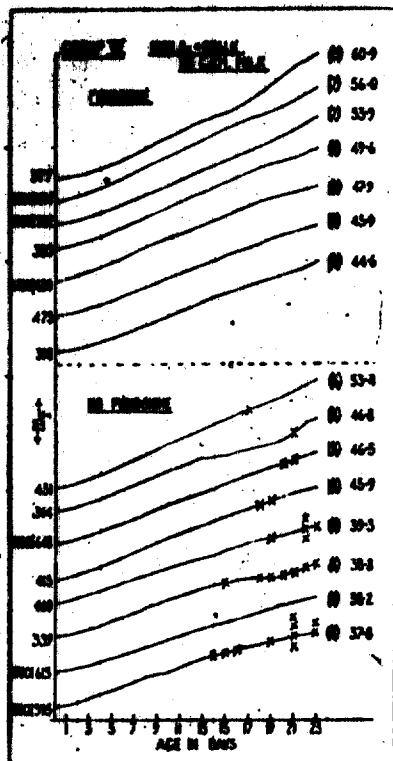


CHART II.—The weight graphs in the upper half represent litters from does which received a daily supplement of 40 microgrammes of pyridoxine from parturition. The graphs in the lower half represent litters from does which received no pyridoxine. "Stock" indicates that the doe was a stock rat placed on the experimental diet two weeks before mating.

high B_6 + chalk, from which it will be seen that the previous findings are amply confirmed. Seven out of eight litters showed the characteristic convulsive fits, several of them having repeated attacks. Although fits were not actually observed in the eighth litter, it seemed probable that they may have occurred (possibly overnight) when no observer was present, for it was noted that the general condition of the young in this litter at weaning was very similar to that of another litter in the group in which fits had been observed day after day. Moreover, the weaning weights of the young in these two litters were practically identical—38.3 and 37.8 g.

Pyridoxine Supplement.—Seven does in this group were given a pyridoxine supplement of 40 microgrammes daily from parturition, and none of their litters showed convulsive fits. The young progressed quite normally, and the average weaning weight was 49.7 g., as against 41.9 g. in the section of the group which received no pyridoxine supplement. In this experiment also the group with medium B_6 + chalk was approximately equal to that with high B_6 and no chalk. In each of these groups 2 litters showed fits, and again not a single fit was observed in litters the mothers of which received pyridoxine supplementation.

It may be noted that a slight difference is observable through all the groups in this test when compared with the corresponding groups in the previous breeding test. Fresh supplies of flour, casein, and dried yeast had to be used in the repeat experiment, and it is unlikely that the new supplies would agree exactly with the old in their contents of all the B factors. A decrease in the B_6 content of the basal ration, or an increase

in the B_6 content, or both, would tend to minimize the lack of balance of the B constituents, and the findings of the second test are consistent with such a difference in the basal ration. The lack of balance is somewhat less than in the first test, so that the occurrence of fits in any one group is less pronounced than in the same group of the previous experiment. Thus in Group III (high B_6 with no chalk) only 2 litters showed fits, although 5 litters were affected in the previous test, and no instance of fits was noted in the second test in either Group II or Group IV. But the worst groups come in the same order as before, and the fits were entirely prevented in all groups when pyridoxine was given.

Comment

It seems abundantly clear, therefore, that pyridoxine deficiency was the cause of the symptoms observed, that this deficiency was induced by excess of vitamin B_6 , and by excess of chalk, and that the effect was enhanced when both vitamin B_6 and chalk were present in excess. Patton and colleagues (1944), who used two pyridoxine-deficient diets in their experiments, one containing much higher amounts of thiamine, riboflavin, and pantothenic acid than the other, found fewer spontaneous seizures in litters from mothers receiving the smaller amounts of the B vitamins. This is in accordance with our findings in regard to B_6 .

While it is a deficiency of B_6 that has become primarily obvious in our experiments, it seems likely that deficiencies of other B factors may also have been induced by the excess of B_6 . It happens that pyridoxine deficiency produces this spectacular effect in the young rats, and thus can be very readily recognized when other deficiencies may be overlooked. As a matter of fact, skin lesions, which developed during the mating and lactation periods in practically all the females of Groups V and VI, indicated the presence of some deficiency other than that of pyridoxine. The lesions of the extremities which are said to be characteristic of pyridoxine deficiency did not appear, but there was loss of hair on parts of the body, which might possibly be indicative of a B_2 deficiency, and numerous body sores. The bald patches occurred mainly on the forehead and round the ears, and in some cases the under side of the body was practically denuded of hair. The body sores took the form of isolated septic spots, with loss of surrounding hair. György (1934) and others (e.g., Chick *et al.*, 1935) have shown that if the diet of rats is deficient in both B_2 and pyridoxine the flacid dermatitis specific for B_2 deficiency does not become evident until B_6 is supplied. The amount of dried yeast in the diet was intentionally kept at a somewhat low level, to avoid masking any adverse effects of the deficiencies of vitamin B_6 . It is thus possible that the supply of B_6 was suboptimal, and liable to be converted to a definite deficiency by excess of B_6 , just as happened with pyridoxine.

The high dose of vitamin B_6 given in our experiments is, of course, far in excess of the amount likely to be found in any ordinary human diet, and the amount of pyridoxine in an ordinary mixed diet will probably be such that there is little risk of a deficiency being induced by the amount of B_6 present. Danger may lie, however, in the present-day tendency to prescribe vitamin B_6 somewhat indiscriminately, as a dietary adjunct and to give large doses of B_6 orally or by injection in the treatment of various diseases. It is precisely in such cases, in which the patient is probably on invalid diet that is liable to be unbalanced, that a large excess of B_6 may entail unexpected and dangerous results. In the case described by Brandstrup, for example, the patient, a chronic dyspeptic treated with ulcer diet, was given large injections of vitamin B_6 , totalling 220,000 i.u. in 3 weeks. When signs of pellagra developed, treatment with a preparation containing the entire B complex permitted recovery. Apart from the development of pellagra, untoward symptoms of various kinds have been recorded after dosing with vitamin B_6 . Steinberg (1938), who treated cases of chronic arthritis with large doses of vitamin B_6 , records that in a few patients vitamin B_6 therapy caused "typical lesions of herpes zoster," irritation of the peripheral nerve plexes, and spasm of smooth muscle. The pain and irritation ceased when B_6 therapy was withdrawn. Lechner (1943) also describes two cases in which injection of pure vitamin B_6 over long periods produced unfavourable effects. In one case the symptoms resembled those of thyroid over-

damage, and included insomnia, headache, giddiness, and palpitation. It may well be, in view of our results, that such downward effects are evidence of some vitamin deficiency induced by the large excess of the single vitamin.

Little is known as yet regarding human requirements of pyridoxine, but some results recorded by Spies, Bean, and Ashe (1939) are of interest. They found that 4 patients who had been treated successfully for pellagra and beriberi, but who remained on their deficient diets, complained of such symptoms as extreme nervousness, insomnia, irritability, abdominal pain, weakness, and difficulty in walking. All the symptoms disappeared within 24 hours of an injection of 50 mg. of pyridoxine. Later, Spies, Ladisch, and Bean (1940), studying the urinary excretion of B₆ in human subjects, found indications of B₆ deficiency in patients suffering from other clinical deficiency diseases. It is thus evident that pyridoxine deficiency can arise in man under certain circumstances.

It is becoming increasingly recognized that in the treatment of pellagrins with nicotinic acid it is essential to provide other members of the B complex and to prescribe a liberal and well-balanced diet. Our experiments would suggest the necessity for adopting a similar procedure for other B factors, and in particular, when B₆ therapy is indicated, for supplying the whole B complex instead of the single vitamin. It was suggested in the previous paper (Richards, 1943) that the improvements effected in a poor human diet by means of such simple supplements as inorganic calcium, milk, and dried yeast provided a useful pointer for the post-war feeding of the starved populations in Europe. The present results emphasize the need for caution in any attempt to improve the diet of these populations by indiscriminate addition of large supplements of single synthetic B vitamins.

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Phosphorylation of Thiamine in the Intestinal Wall During Absorption in vivo

Several experiments in vitro¹⁻³ have shown that intestinal tissue is able to phosphorylate thiamine. The possibility that thiamine is phosphorylated during its intestinal absorption has been suggested by some authors^{4,5} and denied by others⁶. However, a clear experimental evidence of the relationship between thiamine phosphorylation and its intestinal absorption has never been produced, although MACHIDA⁷ found some thiamine phosphates in the wall of isolated intestinal tracts of the rat after incubation with thiamine.

Recently, VENTURA and RINDI⁸ were able to show an active transport of thiamine by the everted intestinal sacs of the rat in vitro, and put forward the hypothesis that the underlying mechanism of transport could be thiamine phosphorylation.

Here we will refer to some results we obtained in an in vivo study of thiamine phosphorylation during the intestinal absorption of equivalent amounts of thiamine hydrochloride and thiamine-propyl-disulphide (TPS), a

well-known thiamine derivative rapidly absorbed and completely transformed into thiamine by the intestinal mucosa⁹⁻¹¹.

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Thiamine phosphate content of rat intestinal wall during thiamine absorption. Mean \pm S.E.

Compound administered	Dose ^a mg	No. of rats	Content ^b , μ g/g wet tissue			P ^c
			TMP	TDP	TMP + TDP	
Saline (controls)	—	5	0.25 \pm 0.03	0.91 \pm 0.11	1.16 \pm 0.09	
T	0.1	3	0.31 \pm 0.02	1.69 \pm 0.38	2.00 \pm 0.36	0.05 \pm 0.02
TPS	0.1	5	0.47 \pm 0.17	1.36 \pm 0.16	1.83 \pm 0.19	0.05 \pm 0.02
T	1.0	3	0.14 \pm 0.01	2.47 \pm 0.47	2.61 \pm 0.34	0.01 \pm 0.01
TPS	1.0	6	0.65 \pm 0.23	4.15 \pm 0.50	4.80 \pm 0.44	< 0.001

T = thiamine; TMP = thiamine monophosphate; TDP = thiamine diphosphate; TPS = thiamine propyldisulphide. ^a Dissolved in 5 ml of saline. ^b Calculated for TMP + TDP, with Student's *t* test, in comparison with the controls. ^c Expressed as thiamine chloride hydrochloride.

The intestinal absorption was studied by a modification^{11,12} of the Cori technique on the small intestine in situ. The rats were sacrificed 2 h after the introduction into the intestine of the compound used, dissolved in 5 ml of saline, since we found in other experiments that, at that time, the amount of thiamine phosphate was the greatest¹¹. The whole intestine was then rapidly taken, emptied, blotted on filter paper and finally homogenized for 3 min by an Ultra-Turrax mod. 45/6 homogenizer in 5% trichloroacetic acid. The final pH was 0.80–0.85.

When we used thiamine hydrochloride, the thiamine phosphate content was calculated by the difference between total thiamine and free thiamine, both determined by the thiochrome method¹³, following the modifications suggested by MICKELSEN et al.¹⁴ in order to lower the blanks.

When we used TPS, an analytical procedure (to be published in extenso¹⁵) was devised, which allowed the simultaneous determination of free and phosphorylated thiamine as well as of TPS by the thiochrome method of MICKELSEN et al.¹⁴.

Recovery experiments, adding to intestinal tissue known amounts of thiamine, TPS and/or thiamine diphosphate, always gave reliable results; no transformation of TPS was ever noticed.

The phosphorylated thiamine content of the intestinal wall, expressed as % of the control content determined after introduction into the rat bowel of 5 ml of saline alone, during absorption of different amounts of thiamine hydrochloride or TPS is shown in the Figure.

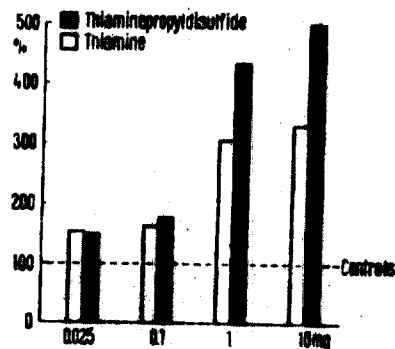
The analysis of variance of the results of the determinations showed a significant difference among the doses; between the two compounds, the difference was significant only for the doses of 1 and 10 mg.

A confirmation of these results came from some experiments where the specific method of RINDI and DE GIUSEPPE¹⁶ was used to determine separately thiamine monophosphate and thiamine diphosphate in the intestinal wall during absorption of 0.1 and 1 mg of thiamine hydrochloride or equivalent amounts of TPS. The results (Table), which are in good accordance with those shown in the Figure, specify that the increase of the phosphorylated thiamine content in the walls was essentially due to thiamine diphosphate. Here again, the increase during absorption of 1 mg of TPS was significantly greater than that during absorption of 1 mg of thiamine hydrochloride ($p < 0.001$).

In conclusion, during the intestinal absorption of thiamine in vivo, a significant increase of phosphorylated thiamine (thiamine diphosphate) in the intestinal wall was demonstrated, both with an indirect and with a direct specific analytical procedure, 2 h after the introduction of equivalent amounts of thiamine hydrochloride

or TPS. The increase after administration of 1 and 10 mg of TPS was higher than after the equivalent quantity of thiamine.

Some in vitro experiments with labelled thiamine are now in progress to investigate further the relationship between thiamine phosphorylation and thiamine intestinal absorption.



Thiamine phosphorylated contents of rat intestinal wall, expressed as percentage of the control content, during absorption of different amounts of thiamine hydrochloride or thiamine propyldisulphide.

Riassunto. 2 h dopo l'introduzione di tiamina cloridato o di tiaminpropyldisolfuro nell'intestino tenue di ratto in vivo si trova un aumento significativo di tiamina fosforilata nella parete intestinale, essenzialmente a carico del tiamindifosfato.

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Istituto di Fisiologia umana, Università degli Studi di Ferrara (Italy), February 21, 1966

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EFFECT OF MASSIVE DOSES OF RIBOFLAVIN, AND OTHER VITAMINS OF THE B GROUP, ON SKIN CARCINOGENESIS IN MICE

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It came to our notice that a large group of Strong A mice in another institute fed on a diet known as "P.R.M." unexpectedly failed to develop skin tumours in response to twice weekly applications of 0.1 ml. 0.1 per cent 3,4-benzopyrene for over one year. Because P.R.M. diet has a high content of vitamins of the B group, and because there are certain theoretical reasons for believing that riboflavin, in particular, might influence carcinogenesis, the experiments described below were undertaken.

Previously Boutwell, Brush and Rusch (1949) investigated the effect of different levels of dietary vitamins of the B group on skin carcinogenesis by 3,4-benzopyrene. They reported a somewhat lower incidence of tumours in a group given a diet low in all B vitamins, but saw no difference in groups receiving diets rich in all, or individual, B vitamins. However, in their experiments the highest dietary level of riboflavin was less than 100 μ g. per mouse per day. Moreover, their treatment with benzopyrene (0.3 per cent in benzene twice weekly, volume of solution not stated) was probably vastly in excess of that necessary to induce tumours, and a small inhibitory effect may have been swamped.

In the experiments reported below, the dose-levels of riboflavin were much higher and the concentration of benzopyrene (in Experiments II and III) much lower.

MATERIALS AND METHODS

Mice. "101" strain mice of both sexes were used in all experiments. Animals were vaccinated on the tail at the age of 6-8 weeks, as a precaution against ectromelia, and began treatment at 8-10 weeks of age.

Chemicals. 3,4-Benzopyrene (BP) and 9,10-dimethyl-1,2-benzanthracene (DMBA) were obtained from L. Light and Co. and used without further purification. Croton oil was obtained from Messrs. Stafford Allen and Co., Wharf Road, London, N.E. in 1953 and thereafter stored in the dark at room temperature. Acetone (Analaar grade) and Thiamine B.P. were obtained from British Drug Houses. Riboflavin B.P., Nicotinic Acid B.P. and Pyridoxine hydrochloride B.P.C. were obtained from "Roche".

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It was concluded that P.R.M. diet, or thiamine in the drinking water of mice on R.I.F. diet, may have protected mice slightly against developing papillomas in response to treatment with DMBA and croton oil.

TABLE I.—*Effect of Riboflavin, Thiamine, Pyridoxine and Nicotinic Acid on the Induction of Skin Papillomas by a Single Application of DMBA followed by Repeated Applications of Croton Oil*

Group	Diet	In drinking water	Applications to skin (begun 4 weeks after starting on diet + vitamins in drinking water) in 0.15 ml. acetone once	Incidence of papillomas at end of croton oil treatment		Incidence of papillomas per survivor one month after end of treatment		Average weight gain during experiment (g.)
				Survivors	Mice with papillomas	Average papillomas per survivor		
I		—	225 µg. DMBA	19	17	12.5	10.0	5.7
II		Riboflavin—2 mg. per mouse daily		19	19	12.5	10.7	5.4
III	R.I.F.	Thiamine 0.2%	Then after 3-week interval	20	18	9.3	7.3	6.4
IV		Pyridoxine 0.04%		18	17	14.1	9.1	5.8
V		Nicotinamide 0.2%	0.25 ml. 0.1% croton oil in acetone once weekly for 15 weeks	19	17	14.6	12.1	6.1
VI	P.R.M.	—		19	18	8.4	7.5	7.4

Experiment II

After randomization 6 groups, each of 10 males and 10 females, were treated as indicated in Table II. Diets and vitamin treatments were begun 4 weeks before applications to the skin.

Papillomas began to appear in Groups VII and VIII during the 11th week, in Groups IX and X during the 13th week, and in Groups XI and XII during the 17th week.

Treatment with DMBA and BP was stopped after 20 weeks; so too were the injections of thiamine for Group XI in which 12 of the mice had died. All other treatments continued. At the 20th week mice of Group VIII had a lower average number of papillomas per surviving mouse than those of Group VII (4.6 as against 6.5) but the proportion of mice bearing papillomas was the same in the two groups. Of the four BP-treated Groups, Group XII (riboflavin) had a lower incidence of papillomas than the others. In addition Groups VIII, IX and X had 1, 2 and 4 malignant skin tumours, respectively. During the next 13 weeks many more malignant skin tumours arose in all the groups. As soon as the diagnosis of malignancy seemed definite, tumours were removed under ether anaesthesia. By following this procedure it was possible for mice to develop several malignant tumours in sequence. Great care was taken to distinguish new malignant tumours from recurrent growths.

Table II gives the totals of mice which bore malignant skin tumours, and of malignant tumours, in each group. There was no difference in incidence between

TABLE II.—*Skin Carcinogenesis by Repeated Applications of DMBA or BP; the Effect of Diet, Yeast, Thiamine and Riboflavin*

Group	Diet	In drinking water	By subcutaneous injection	By application to the skin	Papillomas at 20th week			Malignant tumours arising before 33rd week	
					Survivors	Mice with papillomas	Total papillomas	Mice with tumours	Total tumours
VII	R.I.F.	—	—	0.2 ml. 0.01% DMBA	19	18	124	17	35
VIII	P.R.M. + Yeast*	—	—	× 2 weekly	19	18	88	16	35
IX	R.I.F.	—	—	0.2 ml. 0.025% BP	18	13	37	12	29
X	P.R.M. + Yeast*	—	—	× 2 weekly†	20	12	42	11	28
XI	R.I.F.	Thiamine 0.2%	Thiamine 2.5 mg. × 2 weekly†	0.2 ml. 0.025% BP × 2 weekly	8	5	12	5	8
XII	R.I.F.	—	Riboflavin 0.25 mg. × 2 weekly	0.2 ml. 0.025% BP × 2 weekly	19	8	13	8	21

* 2 oz. per cage of 10 mice twice weekly.

† Stopped after 20 weeks.

the two DMBA-treated groups. Among the BP-treated groups the incidence was slightly, but not significantly, lower in Groups XI and XII than in Groups IX and X.

Apart from those of Group XI, mice in all groups thrived and gained weight at approximately the same rate. Those in Group XI were underweight when the injections of thiamine were stopped at the 20th week, but thereafter picked up, their average weights approaching those in other groups by the 33rd week.

It was concluded that neither the differences in diet nor the administration of thiamine or riboflavin affected skin carcinogenesis by repeated applications of DMBA; but that injected riboflavin may have slightly reduced the incidence of papillomas at 20 weeks, and of malignant tumours arising before the 33rd week, in mice treated repeatedly with BP.

Experiment III

After randomization 3 groups each of 20 male and 20 female mice began treatment with the diets shown in Table III. Four weeks later all groups began twice weekly applications to the skin of 0.2 ml. 0.025 per cent BP in acetone.

Papillomas began to appear in Groups XIII and XIV during the 11th week of treatment, and in Group XV two weeks later. During the next few weeks Group XV lagged behind the other two in tumour development, but from the 18th week onwards this difference progressively disappeared. Table III shows that by the 23rd week there was very little difference between the three groups. The experiment was therefore abandoned at this point.

It is perhaps of passing interest that the mice of Group XV appeared healthier and were always on the average slightly heavier than mice in the other two groups throughout the experiment. Survival in this group was also much better. However, it is well known that intercurrent infections frequently affect one cage of mice more than another. Also it should be noted that Group XIV was never intermediate between the other two groups in health, weight, or survival. Therefore the apparent beneficial effect of riboflavin in Group XV requires confirmation.

TABLE III.—*Effect of Dietary Riboflavin-level on Induction of Skin Tumours by Repeated Applications of BP*

Group	Diet	Applications to skin (began 4 weeks after start of special diets)	Mice in group	Tumour incidence after 23 weeks				Average weight gain during experiment (g)
				Survivors	Mice with tumours	Total benign tumours	Total malignant tumours	
XIII	41B	0.2 ml. 0.025% BP in acetone twice weekly for 23 weeks	40	30	21	76	6	8.0
XIV	41B + 0.2% Riboflavin		40	28	25	74	6	7.2
XV	41B + 0.6% Riboflavin		38	37	22	71	6	8.2

DISCUSSION

One hesitates to report experiments that give negative or inconclusive results. However, in the field of carcinogenesis there is a tendency to invoke special factors in the diet as being responsible for unexpected results. This was the reason for undertaking the experiments reported, and by reporting them it is hoped that

others will be dissuaded from setting up similar experiments. The inclusion of 0.6 per cent riboflavin in the diet of mice, equivalent to more than 150 mg. per mouse per day, in Group XV (Experiment III) is surely a fairly stringent test of the ability of riboflavin to inhibit carcinogenesis by repeated applications of 3,4-benzopyrene to the skin.

The mystery referred to in the introduction of the failure of Strong A mice to develop tumours in response to applications of benzopyrene remains unsolved. It may have had nothing to do with diet. Alternatively, it may have been due to a factor present in one batch of P.R.M. diet but not in others. The latter explanation is supported by the fact that later batches were apparently less yellow than earlier ones. However, detailed enquiries into the possible differences between batches have consistently failed to provide a basis for planning further critical experiments.

Tannenbaum and Silverstone (1953) with respect to the effect of vitamins in the diet on carcinogenesis concluded:

"Analysis of the findings on the influence of varying the level of B vitamins suggests that wide changes in dietary content, in the range above minimal needs, have little effect on carcinogenesis. At least, this appears to be valid for the induced skin tumour and the spontaneous mammary, lung and liver tumours of the mouse. As deficiency levels are approached there may be some inhibition of carcinogenesis, but in these instances calorie intake and body weight changes may be the major cause of the altered response."

This conclusion is not changed by the results of the experiments reported here.

SUMMARY

The administration of massive doses of riboflavin to "101" strain mice had no more than a slight inhibitory effect on their development of skin tumours in response to repeated applications of 3,4-benzopyrene. Additions of other vitamins of the B group had no definite effect at all.

This work was supported by a block grant from the British Empire Cancer Campaign for which I am greatly indebted. Gratitude is also due to Dr. G. Calcutt, Mount Vernon Hospital, Northwood, Middlesex, for advice and provision of certain materials, and to Miss Celia de Mengel and other members of the staff of the Cancer Research Department, London Hospital Medical College, for assistance.

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BAVCH, H. M.—(1950) *J. Hyg., Camb.*, 48, 171.
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TANNENBAUM, A. AND SILVERSTONE, H.—(1953) *Advanc. Cancer Res.*, 1, 451.
THOMSON, W.—(1936) *J. Hyg. Camb.*, 36, 24.

Rowett Institute Formula (R.I.F.) diet prepared according to the formula of Thomson (1936) was obtained from the North-Eastern Agricultural Co-operative Society Limited, Bannermill Place, Aberdeen.

Diet 41B prepared according to formula of Bruce and Parkes (1949) as modified by Bruce (1950) in unpelleted powdered form was obtained from Messrs. Dixon, of Ware, Hertfordshire.

P.R.M. diet.—The P.R.M. diet used in experiments referred to in the introduction, was described to us as visibly yellow. Diet of the same formula¹ and specifications was obtained from the same supplier (Messrs. Dixon of Ware, Hertfordshire). However, it was not noticeably yellow in appearance.

Yeast of various brands was obtained at frequent intervals from local suppliers.

¹ Formula of P.R.M. diet :

	Per cent
Wheat meal (millable grain)	20
English fine wheatfeed	20
Finely ground oats (thin husk)	20
Maize meal (yellow variety)	9.4
Barley meal (plump grains)	5
English white fish meal (66 per cent albuminoids)	5
Meat and bone meal (80 per cent protein)	8.7
Dried skim milk	7.5
Dried unextracted diastase yeast	2.5
Vitamin premix	1.25
Mineral supplement	0.65

Vitamin Premix :

	Per pound
Vitamin A (stabilized)	140,000 I.U.
Vitamin D ₃ (stabilized)	30,000 I.U.
Vitamin B ₂ (riboflavin, nicotinic acid, folic acid)	300 mg.
Vitamin E	900 mg.

Experiment I

After randomization, 6 groups each of 10 male and 10 female mice began treatment with the diets and vitamins shown in Table I. Four weeks later all groups were given a single application of DMBA (225 μ g./0.15 ml. acetone), and after a further 3 weeks the first of a course of 15 once-weekly applications of 0.25 ml. 0.1 per cent croton oil in acetone. After 6 weeks of croton oil treatment papillomas began to appear in all the groups. Their incidence rose steadily, though slightly less quickly, in Group III (given R.I.F. diet plus thiamine in the drinking water) and Group VI (given P.R.M. diet) than in the other 4 groups. Table I shows the incidence of papillomas one week after the end of croton oil treatment, and 4 weeks later.

At the beginning of the experiment the average weight of mice in all groups was 20.7 g. At the end of the experiment Groups III and VI had put on rather more weight than the others. The same two groups had a somewhat lower incidence of tumours. The association of a lower tumour incidence in these groups with a higher rate of body weight gain is the opposite of that reported by Tannenbaum and Silvenstone (1953) in experiments where the quantity but not the quality of the diet was varied. However there were such marked differences in the numbers of tumours borne by individual mice that the difference in average tumour incidence did not reach statistical significance.

Sato, S.: EXPERIMENTAL STUDY ON THE TOXICITY AND PHARMACOLOGICAL ACTION OF THIAMINE DERIVATIVES. I. STUDY ON THE TOXICITY, PHARMACOLOGICAL ACTION AND EFFECT OF THIAMINE DERIVATIVES. Tokyo Ikadaigaku Zasshi, Vol. 25, No. 5, pp. 583-603, 1967. Department of Pharmacology, Tokyo Medical College.

I. INTRODUCTION

Recently, vitamins and their derivatives have been used for therapeutic purposes at massive doses, as well as for nutritional purposes. Vitamin B₁ is the most frequently used vitamin, and, with the commercial sale of its so-called active derivatives, the use of this vitamin is rapidly increasing. Under this circumstance, it is imperative to reexamine its toxicity and study its pharmacological actions.

As is generally known, vitamin B₁ (thiamine) has long been regarded as the factor related to human beri-beri and polished rice disease in laboratory animals, and the study on this vitamin was practically begun in Japan. In 1910, Suzuki (1) carried out an investigation on this substance, and called it Oryzanin. Hara of this faculty (2,3) organized the results of his studies on vitamin B₁ in Germany in 1922, and published several papers based on the results.

In 1927, Jansen (4,5) and Donath et al. separated vitamin B₁ from rice bran and crystallized it. In 1936, Williams (6) identified its chemical structure and artificially synthesized the compound. Vitamin B₁ has thence been called thiamine. The thiamine currently used is its hydrochloride or mononitrate, therefore referred to as thiamine hydrochloride or thiamine nitrate. It is also called antineuritic vitamin because of its physiological action.

With regard to its biological fate or action mechanism in an enzymologic sense, since Lohmann's discovery of cocarboxylase in 1937 (7), its action has been known to be basically related to a catalytic mechanism in the decarboxylation reaction based on the fact that the acetaldehyde formation reaction by the decarboxylation of pyruvic acid and the acetyl-CoA and succinyl-CoA formation reaction by the oxidative decarboxylation of pyruvic acid and -ketoglutaric acid, or the transketolase reaction are catalyzed by an enzyme requiring pyrophosphoric ester (co-carboxylase) of thiamine. Enzymologically, free thiamine without pyrophosphate is inactive in internal metabolism, and pyrophosphoric ester of thiamine can be regarded as an active substance.

The state of being an active vitamin particularly in the case of thiamine refers to the state of free thiamine in relation to thiamine deficiency from the nutritional standpoint, and the ester-type thiamine from the enzymological standpoint. Since the incidence of beri beri has dropped notably in recent years, the use of thiamine as a specific nutritional therapy is being reduced. On the other hand, its use as a drug for nonspecific, massive dose therapy has been increased, the illness for which it is applied being expanded to include not only alcohol toxic neuritis, Wernicke's syndrome, and pregnancy neuritis which are attributed to thiamine deficiency, but also the fatigue of nerves and muscles,

arthritis, neuroparalysis, bradyacusia, amyotrophic lateral sclerosis, constipation, acute poliomyelitis, Japanese encephalitis, and myelitis which hardly appear to be related to thiamine deficiency. This has been discussed in the reports published by the Vitamin B study Committee (5) and by Shimazono (9) who conducted a survey on the study of vitamin B. Free Thiamine can be absorbed to some degree even when administered by the oral route, not mentioning other non-oral routes. However, when absorbed from the digestive organs, particularly from the large intestine, it is decomposed by bacterial aneurinase present in the intestine and becomes inactivated. Its absorption and internal retention are also disturbed. Various derivatives have been developed to solve this problem in nonspecific application. Active thiamines are the product of such effort. These derivatives are generally less susceptible to the decomposition by intestinal bacterial aneurinase, can be readily absorbed from the intestines, and are more fat-soluble. Therefore, they exhibit strong affinity toward internal organs, and can be retained for prolonged periods. They are transferred to the blood, particularly red blood cells, and organs in greater quantities, less toxic, and can be readily esterified.

Such derivatives are exemplified by the disulfide type, obtained by the ring opening reaction of the thiamine structure and by forming the S-S bond with 2 molecules of thiamine, or that obtained by forming a side chain in the form of S-CO. Other derivatives contain benzoic acid and mono-phosphate attached to the ethanol at the C-5 position of the thiazol ring (the position at which pyrophosphoric acid is attached in cocarboxylase). The original activethiamine is the disulfide-type, i.e., thiamine disulfide (TDS). Zima et al. (10-13) have carried out a series of study solely on TDS since 1940, and published papers on its antineuritic action, changes *in vivo*, and the duration of its action. Hara et al. (15-17) of this faculty carried out experiments on the toxicity of TDS, and demonstrated that its toxicity is either extremely low or zero. They observed its remarkable effects on thiamine deficiency at doses above a specific level. There are a number of literatures on this subject. Marten (18) assayed the TDS in the urine and tissues, Fujita (14), Rindi (19), and Kawasaki (20) observed the transfer of TDS into the blood and spinal fluid. Petrelli (21) studied its deposition on liver tissues, Rosanov (22) and Ito (23) investigated its intestinal absorption after oral administration. They generally noted that disulfide-type thiamine is quickly absorbed, favorably transferred to blood corpuscles, and tissues, and is highly absorbable. Later, Fujiwara (24-26) and Matsukawa (27) discovered other derivatives of this type, e.g., thiamine allyl-disulfide (TAD), thiamine propyl-disulfide (TPD), thiamine oxyethyl disulfide (TOED), and thiamine tetrahydro furfuryl disulfide (TTFD). The base of these vitamins is TDS. Consequently, the effectiveness of TDS has thence been discussed, and TDS was reexamined in relation to its large-dose administration as an active vitamin.

TDS is sparingly soluble in water, and, if its aqueous solution is to be prepared, acidic solution is necessary. To solve this problem, thiamine disulfide-nitrate (TDS-N), the nitrate of TDS, was newly developed. When the thiamine effect of TDS is to be pharmacologically analyzed, an acidic solvent must be available, and must be examined with regard to its effect. TDS-N is almost completely neutralized upon bonding with two molecules of nitric acid, and its water solubility is thereby enhanced. This should facilitate its pharmacological investigation.

At this faculty, the toxicity test has long been carried out with the growth curve as the basis of analysis. In such experiments, the choice of laboratory animals is important. Particularly, the selection of pure-bred animals is imperative. The author (22) had carried out a study on the number of eggs laid by hens and their growth in northern Nagano-ken for several years, and obtained data of considerable interest. He also utilized the vast amount of basic data on rat growth curves available in this faculty, and comparatively studied thiamine and its derivatives, and their effects on the central nervous system. Part 1 of this paper discusses their acute and chronic toxicities in mice and rats, the effects of thiamine on the entire growth process from hatching to maturity, and their egg laying capacity, and the effects and therapeutic effect of thiamine and its derivatives in thiamine deprived animals.

II. EXPERIMENTAL MATERIALS AND PROCEDURE

Thiamine (TH), thiamine disulfide (TDS) and thiamine disulfide nitrate (TDS-N), supplied from Kongo Kagaku Co., were used. TH and TDS-N are water soluble, but since TDS is sparingly soluble in water, it was dissolved in hydrochloric acid at pH 1.0. In an experiment where the compounds were added to the feed, a suspension solution of each compound was prepared using pure water, or TDS powder was added to powder feed. When TDS was dissolved in hydrochloric acid, the effect of hydrochloric acid was examined in a control experiment.

The laboratory animals used included mice, rats, rabbits, chickens (chicks and hens), and doves.

Since the experiment involved different procedures, the procedures will be described along with the experimental results.

III. EXPERIMENTAL RESULTS

A. COMPARATIVE STUDY OF THE TOXICITY OF THIAMINE DERIVATIVES

1. ACUTE TOXICITY TESTS FOR THIAMINE DERIVATIVES

A) ACUTE TOXICITY IN MICE

Groups of male DD mice weighing about 15 g were subjected to acute toxicity tests. The derivatives were given by the intravenous, intraperitoneal, hypodermic and oral routes, and their LD₅₀ was determined. The calculation of LD₅₀ followed Behrens-Kaerber's method.

1) TH

TH is the base of thiamine derivatives. Since the toxicity of TDS and TDS-N which were synthesized as oxidation products of TH can be obtained from that of TH, the LD₅₀ of TH was calculated.

The LD₅₀ of TH was 125 mg/kg by the intravenous route (caudal), 185 mg/kg by the intraperitoneal route, 570 mg/kg by the hypodermic route, and 2,450 mg/kg by the oral route (Table 1).

When large doses of TH were given, the mice developed tremor, and clonic spasm, subsequently, and the spasm-producing dose and the lethal dose were close.

2) TDS

TDS was dissolved in hydrochloric acid at pH 1.0, and the solution was adjusted to a concentration of 2.0%.

The LD₅₀ was 690 mg/kg by the caudal vein, and 2,500, 4,900, and 26,000 mg/kg by the intraperitoneal, hypodermic, and oral routes, respectively (Table 1).

TABLE 1. ACUTE TOXICITY IN MICE (LD₅₀)

薬物の種類 a 交付方法	TH	TDS	TDS-N	THの溶解に用いたHCl(1%)
d 静脈注射	125 mg/kg	690 mg/kg	390 mg/kg	12 ml/kg
e 腹腔注射	195 mg/kg	2,500 mg/kg	1,850 mg/kg	74 ml/kg
f 皮下注射	570 mg/kg	4,900 mg/kg	3,000 mg/kg	
g 経口交付	2,430 mg/kg	26,000 mg/kg	9,000 mg/kg	

KEY: a, mode of administration; b, type of drug; c, HCl used as the solvent of TH (1%); d, intravenous; e, intraperitoneal; f, hypodermic; g, oral

The hydrochloric acid solution at pH 1.0 used for the preparation of TDS solution was given to the control. The maximum doses given were 18.0, 74.0, and 120 mg/kg by the intravenous, intraperitoneal, and hypodermic routes, but no death occurred at any of these doses. Thus, neither LD₅₀ nor LD₁₀₀ could be obtained even by oral administration (Table 1).

3) TDS-N

The LD₅₀ of TDS-N was 390 mg/kg by the caudal vein, and 1,850, 3,000, and 9,500 mg/kg by the intraperitoneal, hypodermic, and oral routes, respectively (Table 1).

B) ACUTE TOXICITY IN CHICKS AND HENS

The subjects consisted of female white leghorns, weighing 1,960 g in average, 10-day old chicks weighing 54 g in average, 40-day old chicks weighing 296 g in average, and 60-day old chicks weighing 670 g in average. The process of growth was divided into 3 stages, and the LD₅₀ for each stage was obtained and compared with that of TH and TDS. TH and TDS were given by the hypodermic route.

1) TH

The LD₅₀ of TH in chicks and hens are plotted in Figure 1. The values for TH were 725, 675, 850, and 750 mg/kg for the 10, 20, 40, and 60 days old chicks, and hens, respectively (Figure 1). The LD₅₀ was higher in the order of 40 days old chicks = 60 days old chicks > hens > 10-day old chicks > 20 days old chicks.

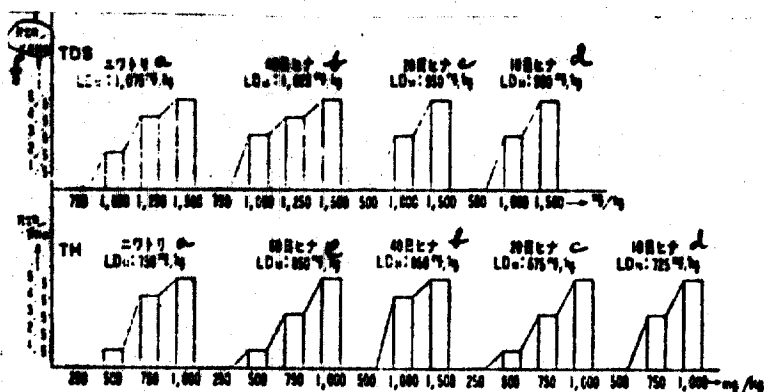


Figure 1. Acute Toxicity of TH and TDS in Chicks and Hens
KEY: a, hens; 40-day old chicks; c, 20-day old chicks; d, 10-day old chicks; f, no. of deaths/number of animals tested

2) TDS

The LD₅₀ of TDS, determined under the same conditions as that of TH, were 950, 950, 1,025, and 1,075 mg/kg for the 10, 20, and 40 days old chicks and hens, respectively. The values are higher in the order of hens 40-day old chicks 20 days old chicks = 10-day old chicks.

Comparing the acute toxicity of TH and that of TDS in chicks and hens, the difference between the 10-day old chicks and hens or grown chicks was 75-175 mg/kg for TH and 125 mg/kg for TDS, the values being not significant. The difference between TH and TDS was 225 - 325 mg/kg. Behavioral changes occurred earlier in the TH group than in the TDS group.

C) ACUTE TOXICITY IN DOVES

Groups of doves, each consisting of 5 weighing about 312 - 390 g, were used for the determination of TH lethal dosage. By the hypodermic route, the LD₅₀ was 725 mg/kg (Figure 2). This is the same as the LD₅₀ in 10-day old chicks. The quantitative relationship for body weight was generally the same in doves and chickens (including chicks).

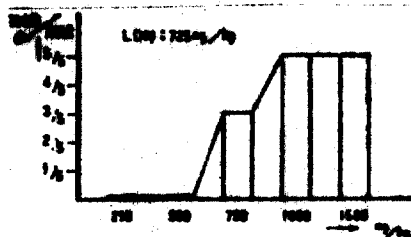


Figure 2. Acute Toxicity (LD₅₀) of TH in Doves
(KEY: a, No. of deaths/No. of doves used)

2. SUBACUTE TOXICITY TEST OF THIAMINE DERIVATIVES

Male DD mice weighing about 12 g were given TDS-containing feeds for 1 month. The oriental powder feed given to the mice contained 0.5 and 5.0% of TDS. Changes in growth curve were studied once daily, and the general condition was also observed.

The 0.5% concentration of TDS is equivalent to 500,000 times the normal dose for human. Figure 3 plots the changes in body weight, in terms of the sum of the weights of 5 in each group. The changes in body weight due to the additive in the feed was manifested in the form of a drop in growth rate for the TDS group as compared to the control group, from the 20th day after the onset of experiment (Figure 3).

3. SUBCHRONIC TOXICITY TEST OF THIAMINE DERIVATIVES

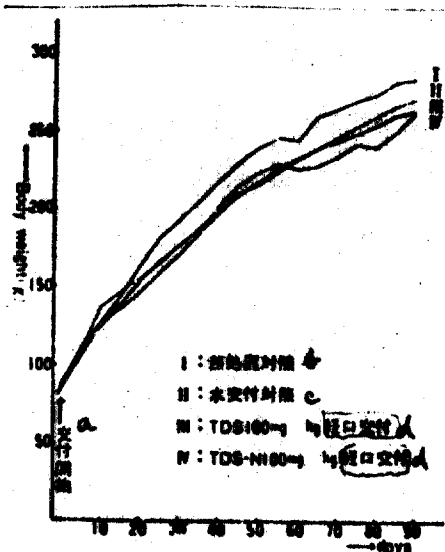


Figure 3. Growth Curves of Rats Given TDS and TDS-N daily for 90 days by the Oral Route (Average of 10)

KEY: a, onset of experiment; b, control without water; c, control with water; d, oral

given water upon demand.

In this faculty, the chronic toxicity test has been carried out for the past several years for food additives, substances which are completely free of toxicity, and the materials which are designed to be consumed for prolonged periods. The test has also been underway for TDS and TDS-N. These tests comprised careful observation of the general condition based on the growth curves. Tissues and blood were also examined for possible manifestation of toxicity.

The rats used for this experiment were Wistar rats, both male and female, weighing approximately 50 g. After observed for more than 1 week, only healthy rats were selected, and were so divided that the average body weight of each group was the same. Each group consisted of 10 rats, and an additional group of the same condition was also provided for supplementing the judgment. The animals were kept in a chamber at the temperature controlled to $22^{\circ} \pm 1^{\circ}\text{C}$. They were individually housed in cages, and

Supposing that a human individual weighing approximately 50 kg is given TDS at 20 mg/day, the dose per kg of body weight would be 0.4 mg/kg/day. The minimal dose in this experiment was set at 1.0 mg/kg, twice the human dose. The dose for TDS-N was calculated by the same method.

1) COMPARISON BETWEEN TDS AND TDS-N

Figure 3 and Table 2 compare the growth curves of male Wistar rats given no drug or water, water, TDS, and TDS-N, both at 100 mg/kg, by the oral route (Figure 3, Table 2).

No significant change occurred for the first 20 days, and slight differences in growth rate between the control and other three groups, i.e., the groups given water, oral TDS, and TDS-N, were noted thereafter. However, there was no difference in weight gain between the water group, the oral TDS group, and the oral TDS-N group except for a slightly lower value shown by the TDS and TDS-N groups on the 90th day. The rats exhibited no abnormal change. TDS produced a transient drop in body weight on the 55th day, but it also occurred to the control group, presumably due to a temporary change in the condition of the cage. These rats were sacrificed immediately after the test by withdrawing the blood from the cervical artery, for the examination of internal organs. The formative components of blood, particularly hemoglobin and blood corpuscles, and the white blood cell count were closely examined, but no change was noted. The 4 groups were also compared

TABLE 2. CHANGES IN BODY WEIGHT OF RATS GIVEN TDS AND TDS-N DAILY FOR 90 DAYS BY THE ORAL ROUTE (AVERAGE OF 10)

a	b	c	d	e	f	a																		
						1	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
TDS	100 mg/kg	22	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23
		88.5	110.0	127.6	140.0	148.7	152.7	156.7	161.2	165.7	169.7	174.1	178.1	182.5	186.5	190.5	194.5	198.5	202.5	206.5	210.5	214.5	218.5	222.5
TDS-N	100 mg/kg	22	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23
		88.5	110.0	127.6	140.0	148.7	152.7	156.7	161.2	165.7	169.7	174.1	178.1	182.5	186.5	190.5	194.5	198.5	202.5	206.5	210.5	214.5	218.5	222.5
TDS	100 mg/kg	22	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23
		88.5	110.0	127.6	140.0	148.7	152.7	156.7	161.2	165.7	169.7	174.1	178.1	182.5	186.5	190.5	194.5	198.5	202.5	206.5	210.5	214.5	218.5	222.5
TDS-N	100 mg/kg	22	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23	23
		88.5	110.0	127.6	140.0	148.7	152.7	156.7	161.2	165.7	169.7	174.1	178.1	182.5	186.5	190.5	194.5	198.5	202.5	206.5	210.5	214.5	218.5	222.5

KEY: a, days; b, temperature; c, control without water; d, control given water; e, body weight; f, before experiment

with regard to the organ weight and volume, which suggested no abnormality beyond the range of physiological fluctuation. Histologic preparations of organs were also examined but no abnormal change was present. Macroscopic examination of organs revealed no edema, hyperemia, tumor, or necrosis.

Figure 4 shows some of the Hematoxylin-eosin preparations of the digestive organ, liver, kidney, and nervous system (cerebrum) which should be more susceptible to the orally ingested drugs (Figure 4).

2) DIFFERENCE IN EFFECT BY SEX

Some drugs exhibit considerable difference in action between male and female, although some shows no difference at all. This aspect was investigated using pure-bred male and female Wistar rats. The conditions of the cage were the same as in the previous tests. The female rats were subjected to the experiment after their vaginal wax was checked. The drug used was TDS-N, and the dosage was 100 and 1,000 times the standard dose.

The results are shown in Figure 5. The growth curves show the same pattern, but the growth rate of the male rats was higher than that of the female rats. There was no appreciable difference between the male control and male TDS-N groups, both indicating normal growth curves. In the female rats, no appreciable difference was noted between the control group and the TDS-N 100 mg/kg group, and the TDS-N 1,000 mg/kg group showed a more favorable curve than the control group, being intermediate between the male groups and other female groups. On an overall basis, the male and female rats showed some difference in growth curve, but there was no difference in general condition, all growing favorably with no incidence of intoxication or no reaction specific to the sexual difference (Figure 5).

The tissues of the female rats revealed no macroscopic change. Their histopathologic examination showed no morbid change.



1. cerebrum. 2. liver. 2. kidney. 4. stomach. 5. intestine

Figure 4. Histopathological Preparations from Rats given TDS and TDS-N Daily for 90 Days by the Oral Route (on the 91st Day). KEY: a, control.

Figure 6 shows some of the ovarian and uterine tissue preparations of TDS-N treated rats, revealing no difference from the control (Figure 6).

2) ORGAN WEIGHT

TDS-N was given to rats and chickens for 90 days consecutively, and the animals were sacrificed by withdrawing the blood on the 91st day. The brain and internal organs were weighed, and changes were carefully studied. The results are given in Table 3, which also provides the data for the TDS group for comparison purpose (Table 3).

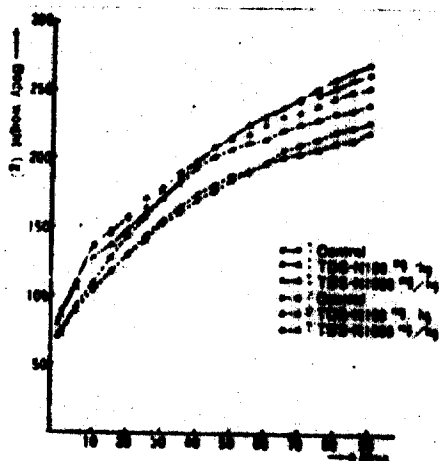


Figure 5. Growth Curves of Male and Female rats Given TDS-N Daily for 90 Days by the Oral Route (Average of 10)

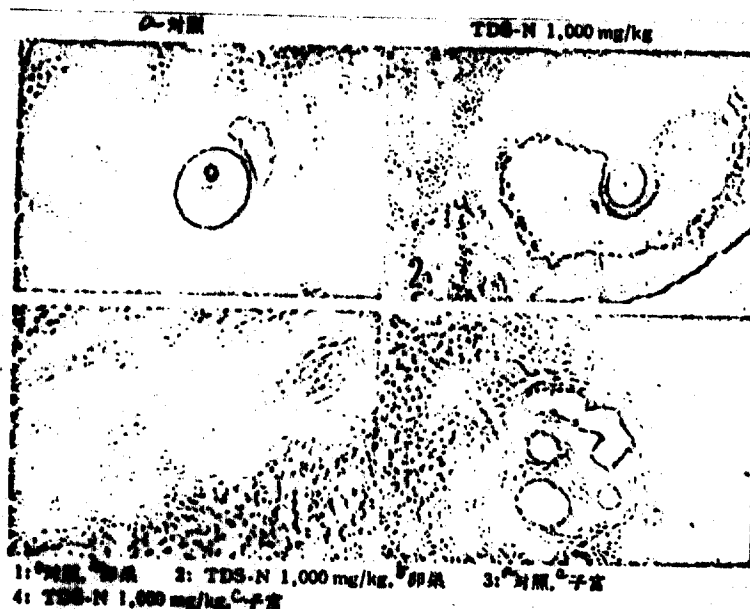


Figure 6. Histologic Preparations of Female Rat Sex Organ After 90 Day Daily Oral Administration of TDS-N.
KEY: a, control; b, ovary; c, uterus

a. RATS

The cerebrum, cerebellum, thyroid gland, thymus gland, heart, liver, and kidneys of the male rats showed no change, and a very minimal decrease was shown by the lung. There were slight differences in the weights of the testis and epididymis among the TDS-N groups regardless of the dose. Because of the small weight of the prostate and seminal vesicle, precise measurement was required, and the results indicated a downward trend within the standard error of 1/100 g. The tail of the TDS-N group grew longer with maturation (Table 3).

The female rats were examined in the same manner. With the exception of the cerebrum and cerebellum, the TDS-N 500 mg/kg group showed generally low levels, and the organ weights of the TDS-N 1,000 mg/kg group were either equal to or in excess of the control levels. On an overall basis, there was no significant change. The uterus or ovaries exhibited no

TABLE 3. ORGAN WEIGHTS OF TDS-N AND TDS TREATED RATS ON THE 91ST DAY (AVERAGE OF 10)

ラットの群別		経口投与量 (10例平均)	性別	体重 (g)	大腸 (g)	小腸 (g)	盲腸 (g)	心臓 (g)	肺 (g)	肝臓 (g)	脾臓 (g)	腎臓 (g)	右腎 (g)	左腎 (g)	右腎 (g)	左腎 (g)	右腎 (g)	左腎 (g)	右腎 (g)	左腎 (g)	右腎 (g)	子宮 (g)	卵巣 (mg)	尾長 (cm)
無処置対照	♂			261.6	1.06	0.27	0.010	0.99	2.46	1.57	9.4	1.04	0.81	0.77	0.022	0.021	0.95	0.95	0.085					16.3
TDS-N 経口投与 100 mg/kg	♂			267.4	1.15	0.26	0.008	1.06	2.01	1.43	9.5	1.11	0.79	0.83	0.020	0.019	0.97	0.99	0.087					17.2
TDS-N 経口投与 1,000 mg/kg	♂			252.6	1.12	0.27	0.010	0.86	2.21	1.49	8.2	0.81	0.77	0.80	0.018	0.015	0.88	0.95	0.037					17.0
TDS-N 皮下投与 10 mg/kg	♂			270.2	1.09	0.24	0.010	0.90	2.01	1.55	9.6	0.90	0.77	0.80	0.019	0.021	0.98	0.99	0.062					16.8
TDS-N 皮下投与 100 mg/kg	♂			278.4	1.12	0.25	0.015	0.97	1.98	1.53	10.4	0.63	0.80	0.94	0.017	0.019	1.12	1.13	0.076					16.4
無処置対照	♀			277.7	1.17	0.34	0.034	0.86	2.11	1.82	10.8	0.78	0.77	0.78	0.030	0.030					0.53	106	15.8	
TDS-N 経口投与 100 mg/kg	♀			220.3	1.13	0.27	0.027	0.83	2.18	1.53	8.3	0.64	0.77	0.79	0.028	0.028					0.82	108	15.5	
TDS-N 経口投与 500 mg/kg	♀			231.2	1.23	0.26	0.032	0.82	2.26	1.58	9.3	0.65	0.79	0.80	0.026	0.029					0.44	107	16.5	
TDS-N 経口投与 1,000 mg/kg	♀			240.6	1.21	0.28	0.028	0.92	1.86	1.57	10.3	0.75	0.81	0.80	0.029	0.029					0.61	127	15.8	
無処置対照	♂			282.7	1.11	0.40	0.013	0.98	1.40	1.28	9.5	0.55	0.98	0.96	0.021	0.020	0.99	0.96	—					17.8
TDS 経口投与 1 mg/kg	♂			287.5	1.08	0.41	0.015	0.97	1.50	1.23	9.8	0.54	0.84	0.85	0.021	0.021	1.06	1.06	—					17.4
TDS 経口投与 10 mg/kg	♂			288.7	1.10	0.39	0.012	0.77	1.45	1.28	8.7	0.51	0.76	0.75	0.019	0.020	1.00	1.01	—					16.3
TDS 経口投与 100 mg/kg	♂			290.8	1.08	0.48	0.015	0.98	1.84	1.42	9.3	0.55	0.90	0.90	0.024	0.022	0.93	0.92	—					16.7

KEY: a, organ and weight on the 91st day (average of 10); b, sex; c, group; d, body weight; e, cerebrum; f, cerebellum; g, thyroid gland; h, heart; i, lung; j, stomach; k, liver; l, spleen; m, kidneys; m', right; m'', left; n, suprarenal gland; o, testis; p, prostate; q, uterus; r, ovary; s, tail; t, control; u, oral; w, hypodermic;

TABLE 4. WEIGHTS OF MAJOR ORGANS (g) OF CHICKENS AFTER 90 DAYS DAILY ORAL ADMINISTRATION OF TDS-N AT VARIOUS DOSES

		a	b	c	d	e	f	g	h	i		j	k	l	m	n	o
										右	左						
P	1	2,700	9.9	46.0	30.0	8.0	80.0	1.4	10.4	5.5	6.3	6.7	0.08	2.48	0.46		
	2	2,900	9.2	38.0	29.0	8.2	76.0	2.3	9.2	7.4	7.3	9.8	0.12	2.70	0.30		
	3	3,300	8.3	38.0	32.0	7.0	78.0	3.2	8.3	7.9	8.7	14.6	0.26	2.50	0.60		
	4	3,100	8.6	38.0	30.0	9.3	77.0	3.1	8.3	7.9	10.6	10.5	0.16	2.60	0.30		
	5	2,900	8.5	38.0	29.0	7.3	87.0	2.7	8.4	8.1	7.2	12.0	0.12	2.80	0.40		
平均		2,900	7.12	35.2	31.0	7.75	86.4	2.54	8.92	7.36	8.02	10.75	0.15	2.61	0.41		
TDS-N 10 mg/kg (口服)	1	2,850	8.9	46.0	32.0	7.3	86.0	2.5	8.2	6.3	7.3	4.7	0.08	2.71	0.40		
	2	2,800	8.3	44.0	29.0	6.1	82.0	1.9	8.4	7.7	7.3	10.5	0.20	2.80	0.30		
	3	2,787	8.2	84.8	31.2	6.8	87.7	2.3	9.4	7.4	7.2	9.2	0.17	2.33	0.32		
	4	3,000	8.4	64.0	30.0	6.8	82.0	2.8	10.3	9.0	10.0	16.0	0.20	1.70	0.30		
	5	2,780	7.6	46.0	34.0	7.0	81.0	2.0	10.7	6.6	4.5	5.6	0.20	2.10	0.30		
平均		2,787	8.28	54.7	31.24	6.8	87.74	2.3	9.4	7.4	7.26	9.2	0.17	2.33	0.32		
TDS-N 100 mg/kg (口服)	1	3,000	8.9	78.0	37.0	7.3	87.0	3.2	8.4	8.6	9.3	6.0	0.20	3.00	0.30		
	2	2,700	8.5	56.0	28.0	7.2	87.0	2.0	8.0	8.6	8.3	14.4	0.30	2.60	0.20		
	3	2,610	8.8	68.8	40.0	7.5	86.0	2.6	9.7	8.8	7.0	9.0	0.30	2.93	0.30		
	4	2,900	8.6	58.0	30.0	7.6	81.0	2.0	8.1	8.9	7.9	8.3	0.20	2.50	0.40		
	5	2,400	7.0	52.0	30.0	7.1	82.0	2.6	8.8	7.5	8.2	10.6	0.18	2.60	0.20		
平均		2,740	8.36	56.16	33.8	7.34	87.0	2.48	8.62	8.48	8.14	9.66	0.24	2.72	0.28		

KEY: a, body weight; b, ovary; c, ovarian tube; d, gizzard; e, stomach; f, liver; g, spleen; h, heart; i, kidneys; j, right; k, left; l, lung; m, thyroid gland; n, cerebrum; o, cerebellum; p, control; q, average; r, oral.

appreciable change. The thyroid gland gave a higher value than that of the male group, but no appreciable difference was noted between the control and the TDS-N groups (Table 3).

b. CHICKENS

The body weight of oral TDS-N group was lower than that of the control group. The weight of the cerebrum and cerebellum combined was the same. The weight of the thyroid gland was higher, and the weights of the kidneys were either higher or the same. There was no appreciable change in the weight of the stomach, spleen, or heart. A slightly lower value was shown by the liver of the TDS-N group. On an overall basis, the values were normal, with no signs of abnormality (Table 4).

4). HEMATOLOGIC FINDINGS

After TDS-N was given to rats and chickens for 90 days consecutively, their blood on the 91st day was collected, and changes were compared between the control group and the TDS-N group. The results are given in Tables 5 and 6 (Tables 5 - 6).

TABLE 5. BLOOD CORPUSCLES AND HEMOGLOBIN COUNTS OF MALE AND FEMALE RATS AFTER 90-DAY DAILY ORAL ADMINISTRATION OF TDS AND TDS-N (AVERAGE OF 10)

C 性 別		D 体 重 (g)	E 血 球 数 ($\times 10^6$)		F 血 色 素 量 (%)
雄	雌		紅血球数	白血球数	
対照	雄	261	871	7,400	98
TDS-N	雄	267	823	6,135	98
TDS-N	雄	252	863	7,630	98
TDS-N	雄	270	768	8,878	98
TDS-N	雄	278	720	9,327	91
対照	雌	227	748	4,915	95
TDS-N	雌	220	792	5,165	100
TDS-N	雌	231	824	5,320	99
TDS-N	雌	240	727	7,790	96
1,000 mg/kg	雌				
対照	雄	263	869	5,571	90
TDS-N	雄	267	676	5,880	92
TDS-N	雄	248	814	5,610	97
TDS-N	雄	280	904	5,480	93

KEY: a, body weight, blood cell count, and hemoglobin level on the 91st day (average of 10); b, sex; c, group; d, body weight; e, blood cell count; f, hemoglobin level; g, red blood cell count (10,000); h, white cell count; i, control with no drug; j, oral; k, hypodermic;

However, the red blood cell counts of the TDS and TDS-N groups were within the range between the upper and lower limits of the control group, showing no significant change.

The female rats showed no appreciable difference in red blood cell count from the control, larger white blood cell count, and the same hemoglobin level (Table 5).

TABLE 6. BLOOD CELL COUNT AND HEMOGLOBIN RATE OF CHICKENS AFTER 90-DAY DAILY ORAL ADMINISTRATION OF TDS-N AT VARIOUS DOSES

C 性 别	D 体 重 (g)	E 红 血 球 数 ($\times 10^6$)	F 白 血 球 数 ($\times 10^3$)	G 血 色 素 (%)
对照	1	400	14,080	70 (%)
	2	380	13,200	63
	3	360	13,760	64
	4	300	13,120	67
	5	310	12,480	60
平均		350	13,328	65
TDS-N 10 mg/kg (口服)	1	320	12,100	62
	2	400	12,800	70
	3	390	13,760	68
	4	400	13,200	72
	5	360	13,100	64
平均		374	13,004	67
TDS-N 50 mg/kg (口服)	1	350	14,000	63
	2	315	13,100	60
	3	400	14,000	68
	4	480	13,400	76
	5	380	13,000	62
平均		374	13,620	66

KEY: a, red blood cell count; b, white blood cell count; c, hemoglobin level; d, 10,000; e, control; f, average; g, oral

a) RATS

The male hypodermic TDS-N group showed smaller red blood cell counts, slightly larger white blood cell count, and the same hemoglobin level. These changes were all within the range of physiological fluctuation (Table 5). However, comparing these values with the TDS group, the hemoglobin level and the white blood cell count were the same, the red blood cell count was higher at a large dose and smaller at a small dose.

b) CHICKENS

The red blood cell count of the control group was an average of 3,500,000 whereas that of the TDS-N group, 3,740,000 - 3,790,000, slightly larger than the control. The white blood cell count was an average of 13,328 for the control and 13,004 - 13,620 for the TDS-N group, with no difference between the two groups. The hemoglobin level of the two was about 66%, with no significant difference between them (Table 6).

B. EFFECTS ON THE GROWTH CURVES OF CHICKENS AND THE NUMBER OF EGGS LAID BY HENS

Chickens begin to lay eggs immediately after maturation, and continue to do so at specific intervals. It has already been mentioned that the health of a hen can be assessed indirectly by the number of eggs it lays, and so are the effects of various drugs (28). The eggs become chicks after a specific period during which they are mechanically warmed, and the chicks mature after a specific period of growth, then begin to lay eggs. The entire process is highly regular, and the selection of chicks with a given level of body weight can be readily achieved. In this experiment, the growth curve from hatching to the egg laying stage was investigated, and the effect of thiamine on their egg-laying capacity was studied.

TDS-N, one of thiamine derivatives, was used. Prior to the administration of TDS-N, the chicks immediately after hatching were divided into groups of 5, and weighed at a specific time daily. Thus, the condition of the chicks was followed on the basis of their growth curve. Figure 7 plots changes in body weight of these chickens, in terms of the average of 5. Table 7 provides the weights of the chick. As is noted in the chart and table, the chickens grew daily at a steady rate. TDS-N was orally given to a group with the least weight gain at 50 mg/kg, and to a group with the second least growth rate at 10 mg/kg from the 115th day after hatching. The remainder, with the highest growth rate, was used as the control.

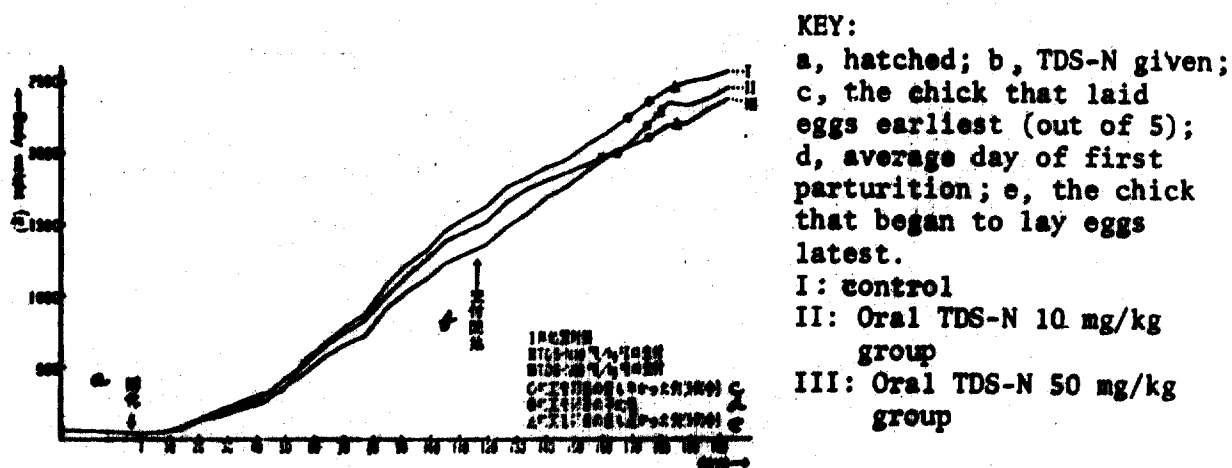


Figure 7. Growth Curves of TDS-N Treated Chickens (egg → hatched → chick → hen) (average of 5)

TABLE 7. BODY WEIGHTS OF CHICKENS GIVEN TDS-N AT VARIOUS DOSES (egg → hatched → chick → hen)
(Average of 5)

第I群		2/VI	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
群别平均 体重5匹		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
第I群	绝食对照	57.0	57.0	55.6	55.8	55.6	55.0	54.8	54.0	54.0	53.8	53.8	53.0	53.0	52.4	51.4	51.2
第II群	TDS-N 10 mg/kg h(经口)	57.4	57.4	56.0	55.6	55.0	54.6	54.4	54.2	54.0	54.0	54.0	53.2	52.8	52.0	51.4	51.2
第III群	TDS-N 50 mg/kg h(经口)	56.8	56.8	55.6	55.2	54.6	54.2	53.8	53.4	53.4	53.4	53.2	52.4	52.4	52.0	51.4	51.2
第II群		18	19	20	21	22	23	24		25	26	27	28	29	30	1/VI	2
群别平均 体重5匹		17	18	19	20	21	22	1		2	3	4	5	6	7	8	9
第I群	绝食对照	51.0	50.0	50.0	49.8	49.4	40.0	38.4		36.0	37.2	42.6	46.2	46.4	47.8	53.2	58.8
第II群	TDS-N 10 mg/kg h(经口)	51.0	50.2	50.2	49.6	49.4	47.0	38.6		36.6	39.4	41.6	47.2	47.8	48.8	50.4	55.4
第III群	TDS-N 50 mg/kg h(经口)	51.0	50.2	50.2	49.6	49.4	39.4	37.2		35.2	35.0	39.6	41.8	4.22	43.6	46.0	49.2
第II群		3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18
群别平均 体重5匹		10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
第I群	绝食对照	61.0	65.4	70.8	78.8	86.0	96.0	103.8	110.8	118.8	128.8	137.6	145.0	154.8	168.2	179.8	179.8
第II群	TDS-N 10 mg/kg h(经口)	56.0	58.6	62.2	65.4	71.2	79.8	88.8	91.8	95.8	105.4	116.0	123.6	132.2	139.2	145.6	152.6
第III群	TDS-N 50 mg/kg h(经口)	52.2	56.0	59.8	63.0	67.0	76.6	81.8	87.2	93.4	100.8	107.8	114.0	120.2	125.4	132.2	136.2
第II群		19	20	21	22	23	24	25	26	27	28	29	30	31	1/VI	2	3
群别平均 体重5匹		26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41
第I群	绝食对照	189.4	196.2	202.2	211.2	219.0	226.4	234.8	241.8	248.8	256.4	260.8	266.2	272.2	279.2	288.6	301.0
第II群	TDS-N 10 mg/kg h(经口)	160.4	168.8	176.2	189.0	197.0	204.2	212.6	218.8	228.0	236.8	246.2	252.8	258.2	264.2	274.8	284.4
第III群	TDS-N 50 mg/kg h(经口)	144.8	150.6	155.4	160.6	167.8	173.4	178.8	185.2	191.6	195.8	203.0	208.0	214.0	220.6	232.8	241.2

KEY: a, group; b, average body weight; c, days; d, month and day of test (1964); e, group I; f, control group; g, group II; h, oral; i, group II; j, hatching; k, onset of TSD-N administration

a 完成日期 1994年4月		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
群别	群别平均 体重 5 匹	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57
第 I 群	起始对照	311.4	329.8	348.2	371.6	382.8	394.4	509.6	429.0	436.0	450.2	464.6	478.0	493.0	512.4	538.2	554.8
第 II 群	TDS-N 10 mg/kg h(经口)	294.8	313.2	332	351.6	363.2	375.2	386.0	404.0	418.0	432.4	447.6	463.8	474.0	490.8	506.6	523.2
第 III 群	TDS-N 50 mg/kg h(经口)	249.8	265.6	282.8	299.0	309.8	321.2	334.6	349.8	365.2	377.2	387.4	400.0	414.2	428.2	445.4	463.2
a 完成日期 1994年4月		20	21	22	23	26	29	1/X	4	7	10	13	16	19	22	25	28
群别	群别平均 体重 5 匹	58	59	60	61	64	67	70	73	76	79	82	85	88	91	94	97
第 I 群	起始对照	571.2	590.0	600.8	614.0	653.0	724.0	778.0	832.0	864.0	934.0	1008.0	1060.0	1126.0	1206.0	1264.0	1308.0
第 II 群	TDS-N 10 mg/kg h(经口)	536.0	551.8	562.0	572.0	632.0	694.0	734.0	794.0	822.0	892.0	940.0	986.0	1062.0	1126.0	1202.0	1244.0
第 III 群	TDS-N 50 mg/kg h(经口)	377.8	493.6	505.8	516.0	562.0	604.0	646.0	692.0	718.0	778.0	836.0	900.0	970.0	1018.0	1074.0	1104.4
a 完成日期 1994年4月		1/X	4	7	10	13	支付 開始	16	17	18	19	20	21	22	23	24	25
群别	群别平均 体重 5 匹	100	1003	106	109	112		115	116	117	118	119	120	121	122	123	124
第 I 群	起始对照	1364.0	1418.0	1448.0	1492.0	1534.0		1560.0	1568.0	1578.8	1580.0	1608.0	1630.0	1663.6	1694.0	1704.0	1720.8
第 II 群	TDS-N 10 mg/kg h(经口)	1300.0	1358.0	1378.0	1406.8	1435.2		1463.6	1470.4	1479.0	1486.4	1500.0	1516.0	1548.0	1576.0	1592.0	1605.2
第 III 群	TDS-N 50 mg/kg h(经口)	1148.0	1196.0	1232.0	1268.0	1284.8		1305.2	1312.4	1317.6	1324.0	1338.0	1360.0	1381.0	1408.8	1422.4	1440.0
a 完成日期 1994年4月		26	27	28	29	30	31	1/X	2	3	4	5	6	7	8	9	10
群别	群别平均 体重 5 匹	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140
第 I 群	起始对照	1733.2	1743.2	1749.6	1758.8	1767.2	1777.2	1785.8	1796.0	1804.0	1813.2	1828.0	1836.4	1849.2	1860.4	1870.8	1880.0
第 II 群	TDS-N 10 mg/kg h(经口)	1628.0	1640.0	1650.0	1662.0	1674.2	1689.2	1704.8	1718.0	1729.2	1738.4	1745.6	1753.6	1760.4	1770.2	1779.2	1782.0
第 III 群	TDS-N 50 mg/kg h(经口)	1433.2	1464.0	1478.0	1499.6	1517.6	1526.0	1534.8	1548.8	1564.0	1578.0	1594.0	1608.0	1626.0	1638.0	1654.0	1676.0

SEE PAGE 14 FOR KEY

2014年12月13日至2015年1月11日		11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
性别	年龄平均 体重5kg	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156
第1组	对照组	1886.4	1892.8	1896.4	1902.0	1908.0	1916.0	1934.0	1948.0	1964.0	1980.0	1996.0	2014.0	2028.6	2040.0	2052.8	2072.0
第2组	TDS-N 10 mg/kg (进口)	1798.8	1806.0	1814.4	1821.2	1828.8	1836.0	1844.4	1850.8	1858.8	1870.0	1876.8	1883.2	1890.0	1901.2	1909.2	1918.0
第3组	TDS-N 10 mg/kg (进口)	1686.8	1694.0	1708.0	1716.4	1721.6	1732.0	1748.0	1766.0	1782.0	1799.2	1810.0	1823.2	1838.4	1852.8	1861.2	1874.0
2015年1月13日至2015年1月11日		27	28	29	30	1/11	2	3	4	5	6	7	8	9	10	11	12
性别	年龄平均 体重5kg	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172
第1组	对照组	2080.4	2088.0	2098.0	2110.0	2122.8	2144.0	2170.0	2190.0	2200.0	2210.0	2221.2	2238.4	2262.0	2284.0	2302.0	2310.0
第2组	TDS-N 10 mg/kg (进口)	1932.4	1944.0	1954.0	1962.4	1971.2	1977.2	1986.0	1994.0	2006.0	2017.2	2028.8	2050.0	2068.0	2082.0	2098.0	2110.0
第3组	TDS-N 50 mg/kg (进口)	1886.0	1888.8	1916.0	1936.4	1954.4	1968.0	1976.0	1984.4	1995.2	2006.4	2015.2	2024.0	2034.8	2049.6	2064.0	2062.8
2015年1月13日至2015年1月11日		13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28
性别	年龄平均 体重5kg	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188
第1组	对照组	2318.0	2326.0	2348.0	2356.0	237.2	2393.0	2410.0	2426.0	2434.0	2440.0	2451.2	2454.0	2460.0	2472.0	2470.0	2468.0
第2组	TDS-N 10 mg/kg (进口)	2124.0	2146.0	2182.0	2214.0	2246.0	2274.0	2292.0	2316.0	2332.0	2344.0	2360.0	2358.0	2360.0	2344.0	2344.0	2338.0
第3组	TDS-N 50 mg/kg (进口)	2086.8	2077.2	2086.0	2095.2	2108.0	2124.0	2140.0	2161.2	2172.8	2183.2	2196.0	2196.0	2186.0	2196.0	2198.0	2190.0
2015年1月13日至2015年1月11日		29	30	31	1/1	2	3	4	5	6	7	8	9				
性别	年龄平均 体重5kg	189	190	191	192	193	194	195	196	197	198	199	200				
第1组	对照组	2474.0	2482.0	2490.0	2490.2	2494.0	2498.0	2506.0	2520.0	2530.0	2546.0	2560.0	2554.0				
第2组	TDS-N 10 mg/kg (进口)	2384.0	2386.0	2384.0	2384.0	2372.0	2378.0	2378.0	2384.0	2386.0	2405.8	2414.0	2432.0				
第3组	TDS-N 50 mg/kg (进口)	2202.0	2212.0	2222.0	2232.0	2244.0	2260.0	2270.0	2300.0	2300.0	2310.0	2332.0	2340.0				

SEE PAGE 14 FOR KEY

The TDS-N 50 mg/kg group indicated a daily increase in body weight from the 1st day of administration, with their rate of weight gain approaching the control level, and, on the 150th day, the curve reached that of the TDS-N 10 mg/kg group (Table 7, Figure 7).

The first laying of eggs occurred during the 40 days between the 160th and the 200th days. The weight of the control chickens at the first laying ranged from 2,240 to 2,520 g, an average of 2,342 g. The TDS-N 10 mg/kg group weighed 2,040 - 2,400 g, an average of 2,200 g at the first laying, and the TDS-N 50 mg/kg group, 2,150 - 2,280 g, an average of 2,225 g. Comparing the outcomes in terms of body weight, the TDS-N groups indicated no significant difference, and the control group showed the body weight 100 g larger than the TDS-N groups at their first laying. Despite the smaller body weight, the TDS-N groups began to lay eggs earlier. This suggests that TDS-N gave favorable effects on the chickens, including the stimulation of weight gain discussed earlier. The symbols on the curves in Figure 7 indicate the average day of the first laying, that beginning to lay eggs earliest within the group, and that laying eggs latest (Figure 7).

C. EFFECTS OF THIAMINE DERIVATIVES ON THIAMINE DEFICIENCY

In order to determine the therapeutic effect of thiamine, thiamine deficiency must be experimentally produced by means of a thiamine-deficient feed, and the effect of the compounds on the condition should be determined.

Mice, chickens, and doves were used in this experiment. As a thiamine deficient feed or low nutritional feed, polished rice was given to the animals for a specific period of time, and the therapeutic effect of thiamine derivatives was examined.

1. MICE

Male DD mice weighing about 12 g were divided into groups, and given Oriental powder feed for mouse (complete diet) and polished rice powder (rice diet). The groups, a total of 6, will be referred to as the 0% TDS group, 0.5% TDS group, and 5.0% TDS group. There were two control, TDS 0% groups.

Each group of mice was weighed as a whole at the same time daily, and changes were studied for 30 days. The results are shown in Figure 8. When the mice died, the weight was still indicated in terms of the sum of the group. Therefore, the changes in body weight due to the difference in feed were considerable.

a. THE MICE FED ON THE COMPLETE DIET

The mice given the complete diet only showed daily increase in body weight from the onset of experiment, and, with a slight variation, it continued to gain up to the 30th day. The general condition was favorable. The curve representing their growth is used as the control (Figure 8 Curve I). The mice given the complete diet + 0.5% TDS showed the same pattern of growth up to the 20th day and a slight decrease in growth rate thereafter.

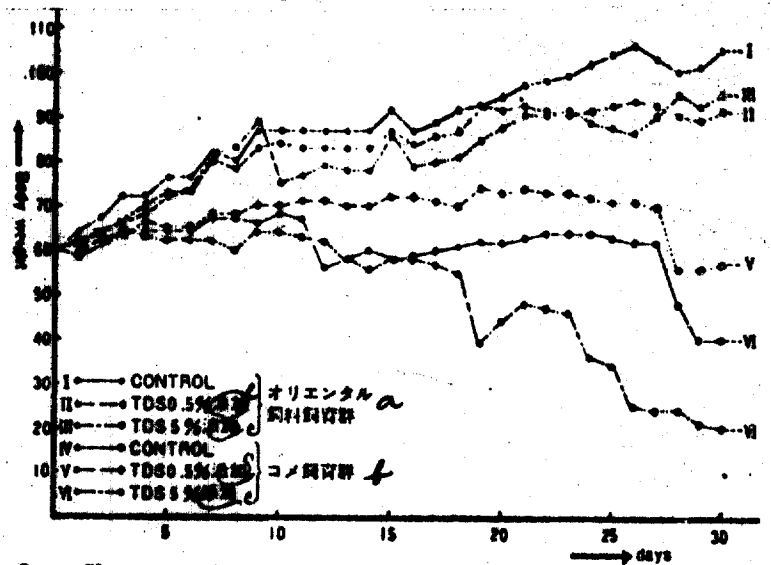


Figure 8. Changes in the Body Weight of Mice Given Complete Diet and Low Nutritional Diet due to TDS
KEY: a, Oriental feed group; b, rice diet group; c, added

Their general condition was favorable (Figure 8 Curve II). The mice given the complete feed + 5.0% TDS showed the same pattern of growth up to the 10th day, but the growth was suppressed thereafter. Their general condition was also favorable (Figure 8 Curve III).

b. THE MICE GIVEN POLISHED RICE DIET

The weight gain of the mice given the polished rice diet only soon became suppressed to a notable degree, with some of the animals dying in 10 days or so. On the 30th day, the surviving showed extremely low growth rate with extremely unfavorable general condition. Their growth curve was used as the control of the TDS groups (Figure 8 Curve IV). The mice given the rice diet + 0.5% TDS indicated a slight increase in body weight up to the 10th day, but a marked drop thereafter, some dying at around the 27th day. Their general condition was somewhat more favorable than that of the control group (Curve V). The mice given the rice diet + 5.0% TDS indicated a slight increase in body weight up to the 20th day, but the growth was suppressed thereafter, with some of them dying (Figure 8 Curve VI).

c. THE WEIGHT OF INTERNAL ORGANS AND THE ORGAN WEIGHT/BODY WEIGHT RATIO OF THE MICE GIVEN THE COMPLETE DIET AND POLISHED RICE DIET WITH AND WITHOUT TDS

The mice were given the diets and were killed on the 31st day. The organs were weighed and the organ/body weight ratio was obtained. The results are shown in Figure 9 and Table 8 (Table 8, Figure 9).

No appreciable change occurred to the cerebrum, cerebellum, heart, kidneys, or stomach. There was a difference in the rates of the liver and the testis for the body weight between the complete diet groups and the polished rice diet groups.

TABLE 8. WEIGHTS (g) OF INTERNAL ORGANS AND ORGAN/BODY WEIGHT INDEXES OF MICE AFTER 30-DAY CONSECUTIVE FEEDING OF TDS-CONTAINING DIETS

		体重 (g)		大 脑	小 脑	心 脏	肺 脏	肝 脏	脾 脏	肾 脏		睾丸	
										右	左	右	左
粉 末 飼 料 飼 育 群	無 毒 照 照	18.8	A	0.31	0.12	0.17	0.17	1.10	0.11	0.15	0.15	0.06	0.06
	無 毒 照 照		B	100	100	100	100	100	100	100	100	100	100
	TDS	18.3	A	0.27	0.10	0.14	0.14	0.98	0.08	0.15	0.15	0.03	0.03
	0.5% 添加		B	150.0	100.0	88.9	87.9	93.1	66.7	115.0	103.6	59.4	62.5
	TDS	17.1	A	0.25	0.09	0.14	0.15	0.98	0.07	0.15	0.15	0.05	0.05
	50.0% 添加		B	147.0	95.0	99.7	101.1	98.3	75.0	112.5	110.8	90.6	93.8
白 米 飼 料 飼 育 群	無 毒 照 照	11.2	A	0.24	0.08	0.15	0.13	0.88	0.06	0.13	0.13	0.08	0.08
	無 毒 照 照		B	2189.	126.7	144.4	131.9	134.5	88.3	146.3	148.2	237.5	243.8
	TDS	12.1	A	0.242	0.09	0.16	0.14	1.27	0.57	0.13	0.14	0.08	0.08
	0.5% 添加		B	119.0	123.3	147.7	127.5	179.3	76.7	140.0	138.6	215.6	218.8
	TDS	9.4	A	0.28	0.10	0.13	0.15	0.97	0.07	0.14	0.14	0.09	0.06
	5.0% 添加		B	298.0	185.0	154.4	168.2	177.6	125.0	186.3	178.3	290.6	293.8

A: average weight (average of 5). B. Organ/body weight index

KEY: a, body weight; b, cerebrum; c, cerebellum; d, heart; e, lung; f, liver; g, spleen; h, kidneys; i, right; j, left; k, testis; l, powder feed group; m, polished rice group; n, control; o, added

TABLE 9. CHANGES IN BODY WEIGHT IN CHICKENS GIVEN VARIOUS DOSES OF TDS-N IN THE POLISHED RICE FEED, AND POLISHED RICE FEED (30 DAYS) (AVERAGE OF 5)

日 数	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
第 I 组	900	884	884	878	886	876	878	880	892	888	878	878	886	884	890
第 II 组	900	894	890	881	892	884	886	892	900	896	900	896	894	892	896
第 III 组	900	892	892	890	892	894	898	904	906	910	914	916	922	924	930
第 IV 组	900	888	892	890	888	896	902	912	922	918	910	928	936	910	944
第 V 组	864	934	1008	1060	1126	1206	1264	1308	1364	1418	1448	1450	1490	1510	1520
日 数	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
第 I 组	890	894	894	900	896	902	907	908	914	920	918	921	926	926	930
第 II 组	902	908	910	922	924	934	932	936	938	910	918	950	918	954	956
第 III 组	932	938	940	916	952	956	960	964	966	970	976	972	972	984	988
第 IV 组	950	958	964	968	972	978	978	984	990	1000	1004	1008	1008	1016	1016
第 V 组	1534	1550	1560	1590	1620	1640	1650	1670	1680	1700	1706	1710	1714	1716	1718

Group I: polished rice group (control); Group II. rice + TDS-N 40 mg/kg
Group III. polished rice + TDS-N 50 mg/kg. Group IV. polished rice + TDS-N 100 mg/kg. Group V. Control

KEY: a, days; b, group

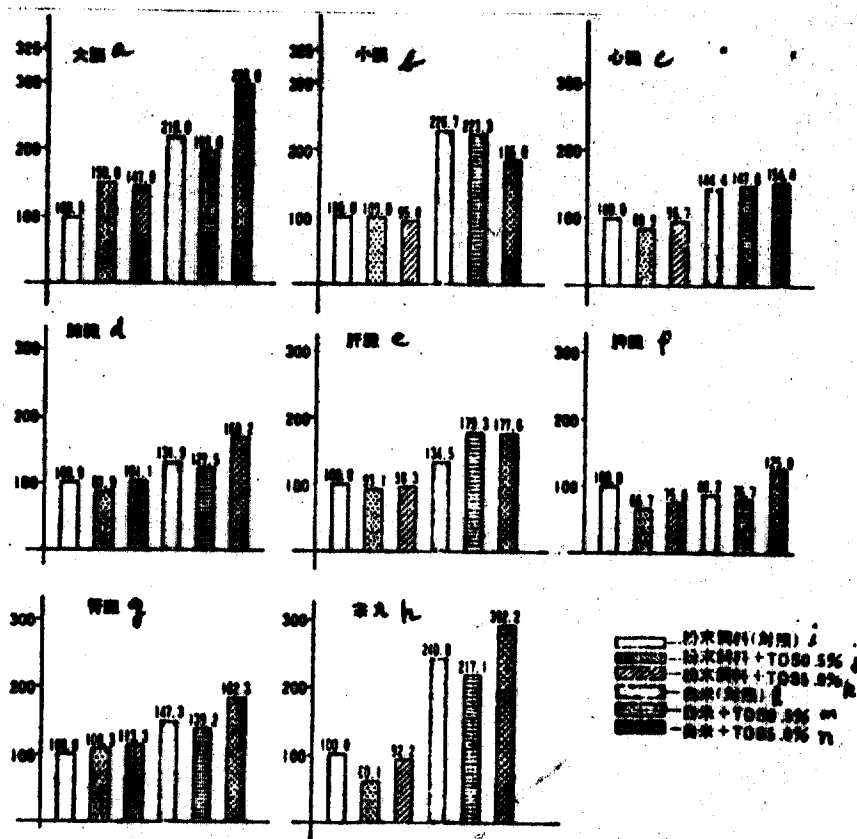


Figure 9. Organ/Body Weight Ratio of Mice on the Complete Diet and the Low Nutritional Diet

KEY: a, cerebrum; b, cerebellum; c, heart; d, lung; e, liver; f, spleen; g, kidney; h, testis; i, powder feed (control); j, powder feed + TDS 0.5%; k, powder feed + TDS 5.0%; l, polished rice (control); m, polished rice + TDS 0.5%; n, polished rice + TDS 5.0%

2. CHICKENS

Chickens weighing an average of 900 g were chosen, and their feed was switched to the polished rice diet. The chickens were divided into 4 groups, and the group given only the polished rice diet was the control. Other groups were given TDS-N in the feed at 10, 50, and 100 mg/kg for 30 days. Changes were studied with their body weight curves as the indicator. The results are shown in Table 9 and Figure 10 (Table 9; Figure 10).

The control group given only the polished rice diet indicated a marked drop in body weight immediately after the switch, with a gradual recovery thereafter. About 20 days later, body weight recovered up to the initial level, and a slight increase was noted thereafter. Comparing the results of other TDS-N groups with this curve, the polished rice diet + 10 mg/kg TDS-N group showed a transient drop in body weight after the onset of experiment, but a gradual recovery to the initial level on the 10th day. The curve gradually rose thereafter. The TDS-N 50 mg/kg group indicated a slightly higher rate of growth but the 100 mg/kg group showed an even higher increase rate than the TDS-N 50 mg/kg group (Table 9, Figure 10). As compared with

the complete diet group, however, the mice given TDS-N in the polished rice diet showed extremely low rate of weight gain, with their growth curve considerably below the level of the complete diet group (Table 9).

3. DOVES

Polished rice was given to doves weighing about 200 - 400 g, thereby producing thiamine deficiency. As a result of the diet, the doves developed hypokinesia in 20 days. They curled up with the neck buried in the feathers and occasionally with tremor. Such condition was even more aggravated in 30 days, with a dull reaction toward external stimuli, and mild convulsion appeared on the 40th day. From the 46th day, the doves were given TDS-N at 10, 50, and 100 mg/kg by the hypodermic route, and the control group was given TD at 50, 100, and 200 mg/kg by the same route, in order to study the therapeutic effects of these compounds on a comparative basis.

The TH reduced the tremor and convulsion in 15 minutes after injection at 50 mg/kg, and brought back automatic movement in 30 minutes. The rate of recovery was higher with higher dose, 100 and 200 mg/kg. The TDS-N exhibited no effect at 10 mg/kg but controlled tremor and convulsion and gradually brought back automatic movement in 15 minutes at 50 mg/kg. At 100 mg/kg, tremor and convulsion diminished within 10 minutes and the birds began to show movement such as peaking. TDS-N exhibited the same or slightly stronger effects, with positive therapeutic effect on the thiamine deprived doves.

IV. CONVULSION AND DISCUSSION

The experimental results will be summarized and discussed below.

1. The acute toxicity of thiamine and its derivatives in mice, chickens, and doves was studied. The results are plotted in Figures 12 and 13. Comparing TDS and TDS-N with TH, the toxicity of TDS and TDS-N given by intravenous injection were 5.5 and 3.1 times that of TH. By the intraperitoneal route, the values were 12.8 and 9.5 times the LD₅₀ of TH, the hypodermic route, 3.6 and 5.3 times, and by the oral route, 10.6 and 3.7 times the value of TH. As evidenced in these numerical values, the acute toxicity of TDS is considerably weaker than that of TH, or even that of TDS-N. The toxicity of TDS-N is intermediate between TH and TDS. Comparing the LD₅₀ by different modes of administration with that for intravenous administration, the LD₅₀ of TH, TDS, and TDS-N were 1.5, 3.6, and 4.7 times higher by the intraperitoneal route, 4.7, 7.1, and 7.7 times higher by the hypodermic route, and 19.6, 37.7, and 23.1 times higher by the oral route. The general tendency of the three compounds was in agreement. By any mode of administration, the value of TH was lower than that of TDS or TDS-N, indicating stronger toxicity. Comparing TDS and TDS-N, the values are relatively close, except for the oral administration in which TDS showed a considerably high value. The site of administration is closely related to the absorption of the compound. When intravenously injected, the compound is transferred directly to the circulatory system, and, when injected from the abdominal route, it is supplied to the blood circulatory system through the peritoneum. By hypodermic injection, the compound must be absorbed through the venous system after entering the tissue. In oral administration, it is absorbed from the intestine, therefore, there should be a difference in absorption between a water soluble derivative and a material which is sparingly soluble in water. As shown in Figure 12,

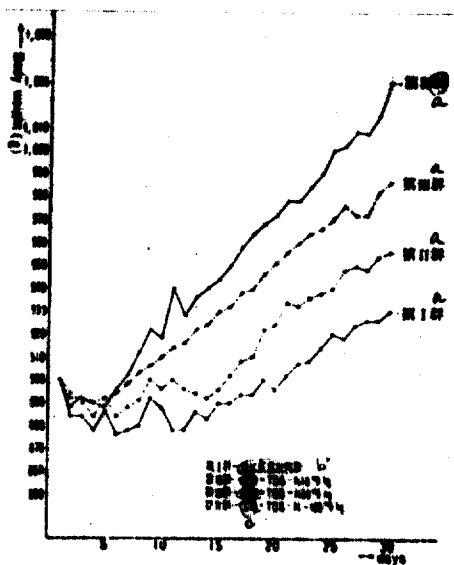


Figure 10. Growth Curves of Chickens Given Polished Rice Feeds with and without TDS-N at Various Doses (300 days) (Average of 5)
KEY: a, group; b, rice diet control group; c, rice.

the active group which do not undergo the exchange of the amine part and the thiazole part which is one of the actions characteristic to aneurinase, as a result of the ring opening in the thiazole part, or are less susceptible to the severence between other pyrimidine part and the thiazole part.

this can be readily assumed from the acute toxicity. TDS is sparingly soluble in water. Its extremely low toxicity when administered by the oral route may be related to this property.

The TDS-N which was solubilized by adding nitric acid to TDS is considerably less toxic than TH, unlike TDS. Judging from these facts, TDS and TDS-N seem to be less toxic than TH, and TDS-N is more readily absorbed from the intestine than TDS. Zima et al. (13) also reported that TDS is least toxic and lasting in effect, and Takagawa (29) observed that TH and TDS are equipotent in mice.

In natural world, thiamine is decomposed by an enzyme with the action of aneurinase, and its efficacy is said to deteriorate upon ingestion, which subsequently affects the efficacy of the compound manifest upon absorption from the intestine. Thiamine derivatives, particularly TDS and TDS-N belong to

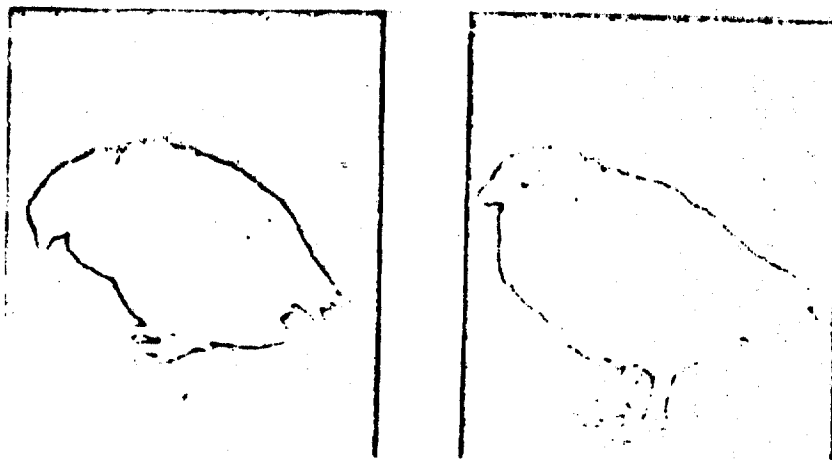


Figure 11. The Effects of TDS-N on the Convulsion Occurring to 40-day old Chick given a Low Nutritional Feed.
(Left: Control (polished rice only, 40 days; tremor and hypokinesia) Right: 30 min. after hypodermic injection of TDS-N at 100 mg/kg)

TDS can be obtained by the oxidation of thiamine in an alkaline state, thereby bonding two molecules of thiamine in the form of thiazole, and contains no bonding which would exhibit any physiological action other than thiamine in its structure. Therefore, the toxicity of such factor needs

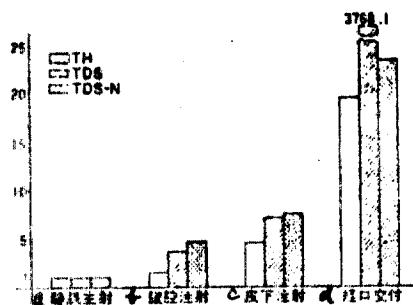


Figure 12. Differences in the LD₅₀ of TH, TDS, and TDS-N by Mode of Administration
KEY: a, intravenous; b, intra-peritoneal; c, hypodermic; d, oral

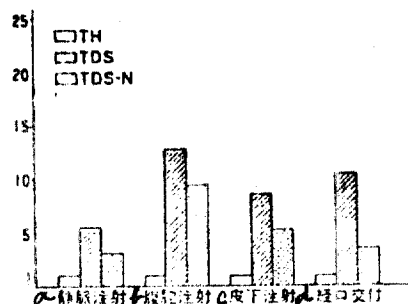


Figure 13. Comparison in LD₅₀ between TH, and TDS and TDS-N.
KEY: a, intravenous; b, intra-peritoneal; c, hypodermic; d, oral

not to be taken into consideration. Therefore, the difference in toxicity between the three compounds is directly attributable to the difference between thiamine, the -S-S-type thiamine disulfide, and its nitrate.

The acute toxicity in mice has been already discussed. In the case of chickens, the toxicity of TH was 750 mg/kg and that of TDS, 1,075 mg/kg, the difference being 325 mg/kg. Thus, the acute toxicity of TDS is weaker. There are many advantages in using chickens for the screening test of thiamine and similar drugs. First of all, a large number of eggs can be obtained and hatched at one time. They can be raised in a simple operation. The process of maturation can be divided into stages. The acute toxicity tests were carried out with 10-, 20-, 40-, and 60-day old chicks and grown chicks, and the relationship between their growth and toxicity was investigated. The difference between TH and TDS in toxicity has already been discussed, i.e., TDS is less toxic than TH. Doves gave values close to those shown by the 10-day old chickens, and they also indicated a similar quantitative relationship with regard to body weight.

2. The subacute and subchronic toxicities of thiamine were tested in mice, rats, and chickens. The difference in toxicity by sex and the effect of prolonged administration were also studied. When TDS was given to mice for 30 days, the changes were confined to a downward trend in growth rate, with no appreciable difference in general condition from the control. Zima (13) compared TH and TDS in a similar experiment, and observed that only TH produced some changes such as hypokinesia, spasm, and protopsis, and paralysis. It was found that repetitive administration of TH made animals hypersensitive, but with the exception of piloerection, TDS caused no change and was less toxic.

In rats, no significant difference was noted between TDS and TDS-N at the same dose. After 90 days of consecutive feeding of the compound, the general condition remained excellent, with no incidence of toxic manifestation. As is shown by its acute toxicity, the difference in LD₅₀ between TDS and TDS-N in oral administration is considerable. However, in a prolonged administration, the difference was not significant, with no difference in organ weight, hematologic findings, or histopathologic impression. This indicates that the toxicity does not necessarily rise as a result of the increased absorbability of TDS in the form of a nitrate. There was no difference in toxicity by sex. Histopathological examination of tissues revealed no abnormal change.

In prolonged use, TH could produce morbid symptoms, but TDS or TDS-N exhibit no abnormality, and, despite their higher absorbability, the toxicity seems to remain without change.

3. The entire process of hatching, maturation, and parturition in chickens was observed, and the effects of thiamine derivatives on the growth of chicks and their laying capacity were studied. It was found that TDS-N exerted favorable effects on the growth and laying capacity. The first parturition occurred earlier in the group given TDS-N than the control. Despite their lower average body weight, they began to lay eggs earlier, with normal general condition or no change in hematologic findings, organ weight, or pathohistological condition. Thus, it is assumed that the compound exhibits a favorable effect on the growth of chickens.

4. The therapeutic effects of thiamine derivatives were studied in thiamine deprived mice, chickens, and doves. Mice were divided into the complete diet group, the thiamine deficient diet group, and the thiamine deficient feed + thiamine (TDS) group. This experiment demonstrated that TDS was therapeutically effective on thiamine deprived mice, and improved the pathological symptoms due to thiamine deficiency in chickens and doves. The thiamine deficiency in these animals was the so-called primary deficiency, and is a form of malnutrition primarily originating in absolute or relative thiamine deficiency. It was not a secondary or conditional thiamine deficiency but the symptoms in animals resembled Wernicke's syndrome. TH is effective toward thiamine deficiency, but it is believed to be decomposed by aneurinase or thiaminase. Kawasaki and Horio (20) observed that thiamine was also decomposed by a heat-resistant factor. However, some of thiamine derivatives are less susceptible to the decomposition by aneurinase. Matsui (30) reported that TDS is one of them. Murata (31) studied the action of aneurinase and maintained that TDS is first converted into thiamine by the action of a reducing factor or hydrolytic factor contained in crude aneurinase, then becomes subject to the action of aneurinase. In his analysis, he assumed that TDS is less susceptible to aneurinase. Nishio (34) also published a similar account.

Meanwhile, Hamamoto et al. (32) compared the effects of TDS and TH in the treatment of white rats suffering from thiamine deficiency, and confirmed that there was no difference between TDS and TH. Takagawa (29) studied the effect of prolonged administration of TDS on thiamine deprived rats, and confirmed that, although there was no appreciable difference in effect between TDS and TH, the TDS group exhibited more favorable growth.

Chiti and Chiarini (33) stated that TDS exhibited a negative reaction in the thiochrome test, but was effective as an active thiamine. The present study using mice produced results that are in agreement with their experimental results, particularly in the effects of TDS-N in chickens and TH and TDS-N in doves. More specifically, TDS and TDS-N are more readily absorbed from the intestine, and are less susceptible to the effect of the decomposing action of aneurinase. In view of the reports by Fujita (35), Rinde (19), Kawasaki (20), etc. on the transfer of TDS and TDS-N to the spinal fluid and blood, the report by Marten (18) on the retention of TDS by animal tissues, Peterlli's observation (21) with regard to the deposition of the compound upon tissues as determined on the basis of the measured value of liver co-carboxylase, and Rosanov's findings on their intestinal absorption following oral administration (22), the compounds seem to be well retained internally, are excellent in duration of action, affinity toward tissues, and stability, and can be readily converted into cocarboxylase.

V. CONCLUSION

The above experimental results are summarized as follows:

1. In the acute toxicity test of thiamine and its derivatives, the toxicities of thiamine disulfide (TDS) and thiamine disulfide nitrate (TDS-N) were appreciably lower than that of thiamine. The particularly low toxicity of oral TDS can be attributed to its poor absorption.
2. Thiamine and its derivatives exhibit nearly zero toxicity in various animals in prolonged administration at the same dose. TDS, and TDS-N are extremely low or nearly zero in toxicity. The compounds exhibited no difference in toxicity by sex.
3. In malnutritional rats and chicks, thiamine and its derivatives exhibited a growth stimulatory action.
4. Both chickens and chicks are the most suitable laboratory animals for testing the effect of thiamine. During the entire process from hatching to egg-laying, TDS contributed to earlier laying of eggs.
5. In various animals, the actions of TDS and TDS-N were similar. They exhibited similar effects on the hematological and histopathological trend. The fact that TDS-N is water soluble is an advantage.

In short, TDS and TDS-N are low in toxicity, exert no adverse effect on the growth of animals, and possess a therapeutic effect.

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サイアミン (Thiamine) 誘導体の毒性及び

薬理作用に関する実験的研究

第1編 Thiamine 誘導体の毒性、薬理作用及び その効果に関する検索

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The Experimental Study on Toxicity and Pharmacological Action of Thiamine Derivatives.

Study 1. Investigation on the Toxicity, Pharmacological Action and Effect of Thiamine Derivatives

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Various thiamine derivatives introduced recently have been more and more frequently used as so-called vitamin B₁ of active type and in massive dose for the purpose of obtaining therapeutic effect. This study was made in order to investigate precisely on their toxicity, pharmacological action and effect: determination of acute, sub-acute and sub-chronic toxicity in various animals including mouse, rat, hen and pigeon; experiments in terms of the growth and development of hatched chicken into adult hen; comparison of the effect on laying of eggs, and investigation of the therapeutic effect on animals fed on low nutrition diet. The findings thus obtained may be summarized as follows:

1. Acute toxicity is significantly lower for thiamine disulfide (TDS) and thiamine disulfide nitrate (TDS-N). TDS is especially low in toxicity when given orally, probably because of poor absorption.
2. Both thiamine (TH) and its derivatives exhibited little toxicity when administered in a certain dose for considerably prolonged time in various animals. Toxicity was markedly lower and almost zero for TDS and TDS-N. No difference was observed between sexes.
3. TH and its derivatives proved to be effective in promoting the growth and development of rat and hen fed on low nutrition diet.
4. TDS tended to accelerate laying of eggs in hen, an experimental animal which is most suitable to the study on the effectiveness of TH both in younger and adult generations.
5. No significant difference was observed in action between TDS and TDS-N in various animals. The same applied to hematological and histopathological findings. TDS-N has an advantage in use in that it is soluble in water.

In short, it may be concluded that thiamine derivatives TDS and TDS-N are of low toxicity, exert little effect on the growth and development of animals and are therapeutically effective.

I. 概 言

近來、Vitamin 及びその誘導体は従来の補養要素としての応用にとどまらず、一方治療薬の効果を目的とした大量投与が行なわれる現況にある。殊に Vitamin B₁ は最もよく用いられ、いわゆる活性型の誘導体が市販されるに及び、益々その使用頻度はたかまりつつある。かかる実情に鑑み、それらの毒性を再検討し、薬理作用を探索することは極めて重要である。

周知の如く Vitamin B₁ (Thiamine) は早くから実験動物での白米病及び人間の脚気における欠乏因子として考えられ、その研究は本邦において始まつたといつて過言ではなく、1910 年鈴木¹⁾ は本物質について検索し、Orysanin と命名した。本及室の原²⁾ も 1922 年横主にドイツにおいて Vitamin B₁ に関する研究をまとめ、数篇の論文にして発表している。

1927 年 Jansen,³⁾ Donath⁴⁾ らは Vitamin B₁ を米糠から分離、結晶化し、更に 1936 年 Williams⁵⁾、らから化学構造を決定及び合成して以来、Vitamin B₁ は Thiamine と呼ばれている。現在使用されている Thiamine はその塩酸塩、または mono 硝酸塩などで、化学名を塩酸 Thiamine 或いは硝酸 Thiamine と呼称し、その生理作用に因んで抗神経炎性ビタミン (antineuritic vitamin) ともいわれている。

Thiamine の生体内運命及び酵素化学における作用機序に関しては、1937 年 Lohmann⁶⁾ らによる co-carboxylase の発見以来、その作用の本質は pyruvic acid の脱炭酸による acetaldehyde 生成反応、pyruvic acid 及び α-ketoglutaric acid の酸化的脱炭酸による acetyl-CoA, succinyl-CoA の生成反応、或いは transketolase 反応が何れも Thiamine の pyrophosphoric ester (co-carboxylase) を要求する酵素によつて触媒され、その作用の本質は脱炭酸反応触媒機構であることが明らかにされている。酵素化学的にみると pyrophosphate をもたない遊離の Thiamine は生体内代謝において不活性で、Thiamine の pyrophosphoric ester は活性物質であるといえる。

さて、Vitamin が活性型を示すということは Thiamine の場合、栄養学的見地では Thiamine 欠乏症に対する遊離の Thiamine が、また酵素化学の見地では ester 型 Thiamine がこれを意味するものである。最近、脚気は著しく減少し、栄養学的特異療法としての応用は益々多くなりつつある。これに反し、治療対象域の拡大による Thiamine 欠乏に基因すると考えら

れるものに alcohol 中毒性神経炎、Wernicke 症候群、妊娠性神経炎がある。更に Thiamine 欠乏症とは考え難い神経や筋の疲労、神経痛、神経麻痺、難聴、筋萎縮性側索硬化症、便秘、或いは急性灰白髄炎、日本脳炎及び脊髄炎などへの応用が増加し、薬物としての非特異的大量療法が多用される現況にあり、ビタミン B 研究委員会報告書⁷⁾、或いは Vitamin B 群研究に関する島岡⁸⁾ の現況の報告がある。Thiamine は遊離の形で用いた場合、非経口的にはもとより、経口的にもある程度吸収される。しかしながら消化管、殊に大腸よりの Thiamine の吸収は大腸に存する細菌の aneurinase により分解されて不活性となり、吸収もやや悪く体内貯留性に難点がある。このため非特異的応用の目的で、これらの欠点を排除した種々の誘導体が合成された。いわゆる活性型 Thiamine がそれである。これらの誘導体は何れも多少の差はあるが、腸管内細菌の aneurinase によつて分解をうけ難く、腸管よりの吸収は Thiamine より良好、且つ脂溶性が大であるため、腸管親和性が強く、体内における貯留時間も長く、血中、殊に赤血球及び臓器中への移行が多いうえ、毒性が低いこと、さらに ester 型になりやすいことなどの諸点が強調されている。

その代表的なものとして Thiamine 構造中 thiazol 核を開環し、2 個の Thiamine を対照的に S-S 結合させた disulfide 型、S-CO の形で側鎖としたもの、thiazol 環の C-5 の位置の ethanol の部分 (co-carboxylase では pyrophosphoric acid がつくところ) に安息香酸、mono-phosphate がつくものなど、多くの誘導体が見出されている。これらのうち、いわゆる活性化の始まりとなったものが disulfide 型の Thiamine、即ち Thiamine disulfide (TDS) で、Zima⁹⁻¹⁰⁾ らは 1940 年来一貫して TDS の研究を行ない、抗神経炎作用、生体内変化、作用の持続性など報告している。また教室の原¹¹⁻¹³⁾ は TDS の毒性実験を行ない、毒性は極めて低いか皆無に近いとし、Thiamine 欠乏の場合、その一定量以上で著しい効果を認めている。また Marten¹⁴⁾ による組織、尿中の TDS 量測定、藤田¹⁵⁾、Rindi¹⁶⁾、川崎¹⁷⁾ らの報告にみられる血中濃度への移行、Petrelli¹⁸⁾ による肝組織への沈着、Roanov¹⁹⁾、伊藤²⁰⁾ らの経口交付による腸吸収など disulfide 型 Thiamine は吸収がはやく、血球、組織への移行性が高く、また体内吸収性が優れている。その後、藤原²¹⁻²³⁾、松川²⁴⁾ らにより Thiamine allyl-disulfide (TAD)、Thiamine propyl-disulfide (TPD)、Thiamine oxyethyl disulfide (TOED)、

Thiamine tetrahydro-thiaryl disulfide (TH) など一連の誘導体が発表されている。これらの Vitamin は TDS を基本とするため、TDS の特性が論ぜられるに限り、大量投与療法における最佳型 Vitamin として再検討されるようになった。

TDS は水に難溶性であり、水溶液を作る際、酸性溶液を必要とする。ここにおいて TDS の硝酸塩である Thiamine disulfide-nitrate (TDS-N) が新しく製造された。即ち TDS は Thiamine 効果を薬理学的に検索する際、試料を必要とし、効果検討の際、その影響を受けなければならない。然るに TDS-N は TDS の硝酸塩の結合によりはほぼ中性化され、水溶性。この点、薬理学的検索に適当である。

本教室においては、数多くの毒性実験を、体重曲線を主軸とした判定法により施行している。これらの実験を行なう場合、動物の選択は重要な意義をもつ。なかんづく一定の純系動物を選択することは必要欠くべからざるものである。余等は長野県北部におけるユワトリの産卵数とその成長に関する基礎的観察を数年にわたり継続し、極めて興味ある結果を得ている。且つ教室のラット成長曲線の膨大な基礎的報告を基に、上記 Thiamine とその誘導体の比較を中心として行ない、また中枢神経系への影響を精細に検索した。本第1篇においてはマウスおよびラットによる急性並びに慢性毒性、ユワトリの孵化しての成長となり、産卵するまでと、それらユワトリの産卵に及ぼす Thiamine 誘導体の影響、また Thiamine 欠乏動物に対する Thiamine 及びその誘導体の影響並びに治療効果を検索し、興味ある結果を得たので報告する。

II. 実験材料及び実験方法

実験材料は金剛化学株式会社提供による Thiamine (以下 TH と略記)、Thiamine disulfide (以下 TDS と略記) 及び Thiamine disulfide nitrate (以下 TDS-N と略記) を用いた。TH 及び TDS-N は水に可溶性であるが、TDS は水に難溶性であるため、pH 1.0 の塩酸に溶解した。しかし飼料に添加さ

合して交付する実験では溶液を用いて懸濁液をつくりて添加、或いは TDS 原質を飼料中にそのまま添加の上使用した。一方、塩酸を塩酸で溶解した場合には塩酸による対照実験を行なって、その影響をも考慮した。

実験動物はマウス、ラット、ウサギ、ニワトリ（以下鶏）と、また成鶏はニワトリと呼称する）及びハトを用いた。

実験方法は多岐に亘るため、実験成績の各項で詳述する。

III. 実験成績

A. Thiamine 誘導体の毒性に関する比較検討

1) Thiamine 誘導体の急性毒性実験

a) マウスによる急性毒性

マウスは体重 15g 内外の DD 系統性マウス 10 匹を 1 群として、静脈、腹腔、皮下及び経口交付により得た LD₅₀ で比較検討した。LD₅₀ の算出は Behrens-Kaerber 法によった。

1) TH の場合

TH は Thiamine 誘導体の基本をなすもので、TH の酸化生成物として合成された TDS、TDS-N の毒性は TH の毒性と比較検討により考察され得るので、TH の LD₅₀ を算出した。

その結果、TH の LD₅₀ は尾静脈内注射で 125mg/kg、腹腔内注射で 195mg/kg、皮下注射で 570mg/kg、経口交付で 2,450 mg/kg であった (第1表)。

なお、TH を大量交付した場合、マウスは振盪、次いで間代性虚脱を出現したが、虚脱出現と致死量は極めて近似であった。

2) TDS の場合

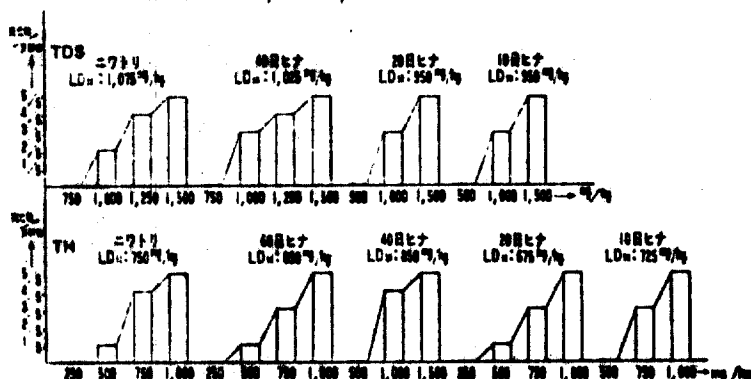
TDS は pH 1.0 の塩酸に溶解し、2.0% TDS 溶液に調製して使用した。

その結果、TDS の LD₅₀ は尾静脈内注射で 690 mg/kg、腹腔内注射で 2,500 mg/kg、皮下注射で 4,900 mg/kg、経口交付で 26,000 mg/kg であった (第1表)。

第1表 マウスによる急性毒性 (LD₅₀)

動物の種類 交付方法	TH	TDS	TDS-N	TH の毒性に用いた HCl (1%)
静脈注射	125 mg/kg	690 mg/kg	390 mg/kg	18 ml/kg
腹腔注射	195 mg/kg	2,500 mg/kg	1,850 mg/kg	74 ml/kg
皮下注射	570 mg/kg	4,900 mg/kg	3,000 mg/kg	
経口交付	2,450 mg/kg	26,000 mg/kg	9,000 mg/kg	

第1図 TH 及び TDS のヒナ及びヒナに対する急性毒性



なお、TDS の溶液調製に用いた pH 1.0 塩酸液を TDS の対照実験に使用した。その結果は尾静脈内注射で 18.0 ml/kg、腹腔内注射で 74.0 ml/kg であり、皮下注射で 180 ml/kg まで交付したが死亡せず、経口交付でも LD₅₀ ないし LD₅₀ は測定出来なかつた (第1表)。

3) TDS-N の場合

TDS-N の LD₅₀ は尾静脈内注射で 300 mg/kg、腹腔内注射で 1,850 mg/kg、皮下注射で 3,000 mg/kg、経口交付で 9,500 mg/kg であつた (第1表)。

B) ヒナ及びヒナによる急性毒性

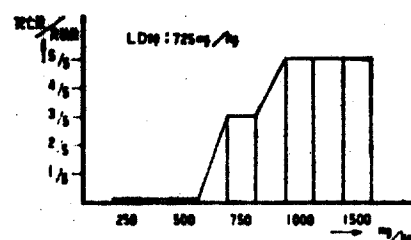
ヒナ及びヒナは白色レグホン系の雌鶏を使用し、孵化後 10日目のヒナ (以下 10 日ヒナと略記) 平均体重 84 g、孵化後 40日目のヒナ (以下 40 日ヒナと略記) 平均体重 296 g、孵化後 60 日目のヒナ (以下 60 日ヒナと略記) 平均体重 470 g、ヒナ平均体重 1,960 g を使用し、幼若から成熟に至る過程を3段階に分けて夫々の LD₅₀ を検索し、TH 及び TDS の比較を行なつた。TH 及び TDS はすべて皮下交付により施行した。

1) TH の場合

ヒナ及びヒナの LD₅₀ は第1図に示す通りであつた (第1図)。即ち、TH の LD₅₀ は 10 日ヒナで 750 mg/kg、20 日ヒナで 675 mg/kg、40 日ヒナで 850 mg/kg、60 日ヒナで 850 mg/kg、ヒナ平均で 750 mg/kg であつた (第1図)。その順位は 40 日ヒナ > 60 日ヒナ > ニワトリ > 10 日ヒナ > 20 日ヒナであつた。

2) TDS の場合

TH と同一条件下における TDS の LD₅₀ は 10 日

第2図 TH のハトに対する急性毒性 (LD₅₀)

ヒナで 950 mg/kg、20 日ヒナで 950 mg/kg、40 日ヒナで 1,025 mg/kg、ヒナ平均で 1,075 mg/kg であつた (第1図)。その順位はニワトリ > 40 日ヒナ > 20 日ヒナ = 10 日ヒナであつた。

ヒナ及びヒナの急性毒性を TH と TDS の両者と比較すると TH では 10 日ヒナと成熟ヒナないしニワトリの間では 75~175 mg/kg の差、TDS では 125 mg/kg で大差なく、TH と TDS では 225~325 mg/kg の差があつた。なお、behavior に変化が見られるのは TH の方が TDS より速やかであつた。

C) ハトによる急性毒性

312~390 g のハト 1 群 5 羽を使用し、TH の致死量を測定した。その結果、皮下注射による LD₅₀ は 725 mg/kg であつた (第2図)。この量はニワトリの 10 日ヒナの量と一致し、体重関係からハトとニワトリ (ヒナも含め) の量的関係は大体一致していた。

2. Thiamine 誘導体の亜急性毒性試験

12 g 内外の DD 系統性マウスを使用し、TDS 添加飼料による 1 ヶ月飼育実験を行なつた。TDS はオリエンタル粉末飼料に 0.5 及び 5.0% 添加し、体重曲線の推移を 1 日 1 回計量し、一般状態と併せて観察し

た。

なお、TDS の 0.5% 添加は人体常用量の 5 万倍相当量である。第 8 図はその推移曲線で、1 群 5 匹の全重量の統計から算出したものである。対照と TDS 添加の飼料組成による体重の変化は交付開始後 20 日以後対照に比し、TDS 交付群は成長率の減少傾向を認めるにとどまった (第 8 図)。

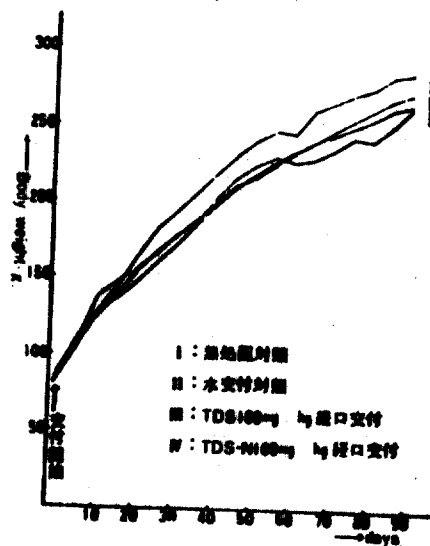
3. Thiamine 誘導体の慢性毒性実験

本実験においては数年来、食品添加物、その他毒性が低いと推定され、特に長期飼育を目的とする物質の慢性毒性の探索を行なっており、TDS 及び TDS-N に対しても慢性毒性の探索を行なつた。これらの実験は飼育曲線を主軸とした一般状態の詳細な観察実験で、組織及び血液を加えて毒性発見の有無を判定している。

本実験に使用したラットは Wistar 系統性及び雌性ラットで、体重 50g 前後のものを 1 週間以上飼育し、健康なもののみを選出し、各群の体重を平均値で統一した。1 群は 10 匹で、同一条件の子飼群を設けて、特定の補助とした。実験に際しては動物専用飼育室を使用し、温度は $23 \pm 1^\circ\text{C}$ に設定した。ラットは 1 飼の cage 中に 1 匹収容し、飲料水は自由に摂取させた。

実験物質中 TDS は鼠にヒト (体重 50 kg) に 1 日

第 8 図 TDS 及び TDS-N を 1 日 1 回連続 90 日間経口交付したラットの体重推移曲線 (10 例平均値)



第 9 表 TDS 及び TDS-N を 1 日 1 回連続 90 日間経口交付したラットの体重推移数値 (10 例平均値)

実験日数	1	5	10	15	20	25	30	35	40	45	50	55	60	65	70	75	80	85	90
対 照 群	22	23	22	21	22	23	22	22	21	23	22	21	22	22	22	22	22	21	22
I 群	80.9	110.6	127.6	143.8	163.7	181.2	192.7	204.8	217.7	229.2	239.0	245.2	253.6	263.7	269.6	273.0	280.9	282.7	
II 群	87.0	106.8	124.3	138.9	153.3	166.4	176.1	186.5	201.7	214.5	223.0	228.4	225.0	227.0	233.2	241.4	239.0	248.4	260.8
III 群	87.8	109.6	137.6	147.2	155.8	165.0	177.6	187.2	198.9	209.6	215.4	223.8	231.0	238.0	243.0	248.0	253.0	259.8	262.4
IV 群	84.2	104.8	127.4	136.2	147.0	159.4	171.0	184.0	196.6	208.6	217.0	225.2	232.2	238.0	245.6	252.4	259.0	265.8	270.0

1回 20mg を交付するとすれば、0.4mg/kg/day となる。そこでこの2倍量の 1.0mg/kg を最低量と定め交付基準量とした。TDS-N の交付量の算出法は TDS に準拠した。

1) TDS 及び TDS-N の比較検討

第3図及び第2表は Wistar 系雄性ラットによる成長曲線を無処置対照、水分強制経口交付対照、TDS 及び TDS-N 各 100mg/kg 強制経口交付の4群に大別し、比較したものである(第3図、第2表)。

実験開始より 20 日後まで有意の変化なく、以後

第4図 TDS 及び TDS-N を1日1回連続 90 日間経口交付したラット第 91 日の
組織組織標本



1: 大肺 2: 肝臓 3: 腎臓 4: 胃 5: 腸

無処置対照群と他の3群、即ち、水分強制経口交付対照群、TDS 及び TDS-N 強制経口交付群との間に、僅少ではあるが、体重増加率に差が認められた。しかしながら水分強制経口交付群と TDS 及び TDS-N 強制経口交付群との間には体重増加に大差は認められず、90 日目 TDS 及び TDS-N 交付群は水分強制経口交付群より僅かに低い値を示したが、ラットの一般状態に異常は認められなかった。なお、TDS は 55 日をすぎると一過性減少を示したが、無処置対照群にも認められる如く、飼育温度等の条件が変つたための一時的現象である。これらの実験使用ラットは実験終了後直ちに頸部動脈より製血致死させて臓器の検査を行なつた。その際、血液有形成分、殊に血色素量ならびに血球数に及ぼす影響、白血球百分比を検査した結果、いずれも有意の変動は認められず、また、臓器重量及び容積の測定から4者を比較検討して、生理的動揺範圍をこえる異常は認められなかった。更に病理組織学的に組織標本による全組織の精細な検査を行なつたが、いずれの組織にも病変は認められなかった。なお、肉眼的所見においても臓器の浮腫、うっ血、腫瘍、壊死は認められなかった。

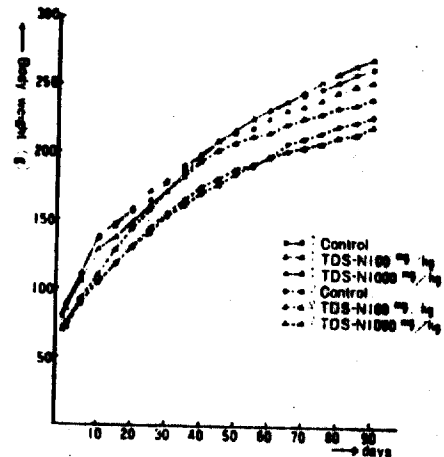
第4図は Hematoxylin-Eosin 染色による上記結果の一部を示したもので、経口交付の影響を受けやすい、消化器、肝、腎及び神経組織(大脳)をとりあげ

た(第4図)。

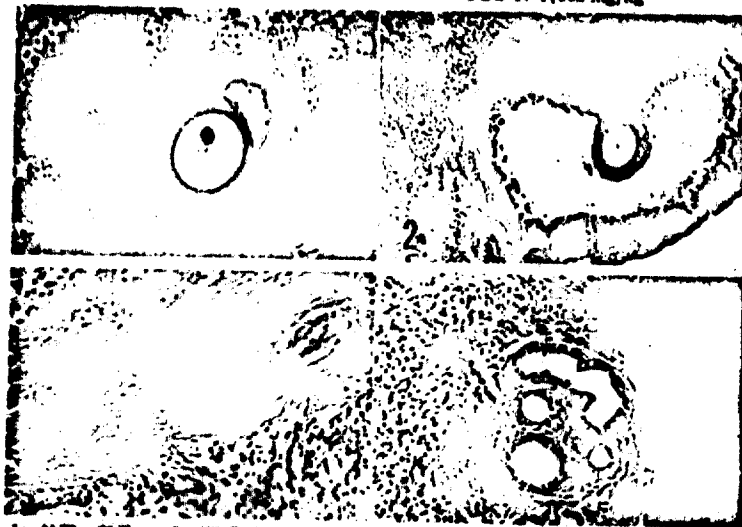
2) 雌雄差に及ぼす影響の検討

一般に薬物の作用は雌雄差により著しく差異を生ずるものもあり、然らざるものもある。そこで Thiamine 系誘導体もこの点を考慮して雌雄差に及ぼす

第5図 TDS-N を1日1回連続 90 日間経口交付した雄性及び雌性ラットの体重推移比較曲線(10例平均値)



第6図 TDS-N を1日1回連続 90 日間経口交付した雄性ラットの性腺の組織標本



1: 対照, 睪丸 2: TDS-N 1,000 mg/kg, 睪丸 3: 対照, 子宮
4: TDS-N 1,000 mg/kg, 子宮

第3表 TDS-N 及び TDS 定付ラットの第 91 日臓器重量比較表 (10 例平均値)

ラットの群別 飼育期間及び91日 経産量 (10 例 平均)	性別	体重 (g)	大腸 (g)	小腸 (g)	甲状腺 (g)	心臓 (g)	肺 (g)	胃 (g)	肝 (g)	脾 (g)	腎		副腎		睾丸		前立腺 (g)	子宮 (g)	卵巣 (mg)	尾長 (cm)
											右 (g)	左 (g)	右 (g)	左 (g)	右 (g)	左 (g)				
無処置群	♂	261.6	1.06	0.27	0.010	0.99	2.46	1.57	9.4	1.04	0.81	0.77	0.022	0.021	0.95	0.95	0.065			16.3
TDS-N 錠 100 mg/kg	♂	267.4	1.15	0.26	0.008	1.06	2.01	1.43	9.5	1.11	0.79	0.83	0.020	0.019	0.97	0.99	0.067			17.2
TDS-N 錠 1,000 mg/kg	♂	252.6	1.12	0.27	0.010	0.86	2.21	1.49	8.2	0.81	0.77	0.80	0.018	0.015	0.88	0.95	0.037			17.0
TDS-N 皮下 10 mg/kg	♂	270.2	1.09	0.24	0.010	0.90	2.01	1.55	9.6	0.90	0.77	0.80	0.019	0.021	0.98	0.99	0.062			16.8
TDS-N 皮下 100 mg/kg	♂	278.4	1.12	0.25	0.015	0.97	1.98	1.53	10.4	0.83	0.89	0.94	0.017	0.019	1.12	1.13	0.076			16.6
無処置群	♀	277.7	1.17	0.34	0.034	0.86	2.11	1.82	10.8	0.78	0.77	0.78	0.030	0.030				0.53	106	15.5
TDS-N 錠 100 mg/kg	♀	220.3	1.15	0.27	0.027	0.83	2.18	1.53	8.3	0.64	0.77	0.79	0.028	0.026				0.52	108	15.5
TDS-N 錠 500 mg/kg	♀	231.2	1.23	0.26	0.032	0.82	2.26	1.58	9.3	0.65	0.79	0.80	0.036	0.029				0.44	107	16.5
TDS-N 錠 1,000 mg/kg	♀	240.6	1.21	0.28	0.028	0.92	1.86	1.57	10.3	0.75	0.81	0.80	0.029	0.029				0.61	127	15.8
無処置群	♂	262.7	1.11	0.40	0.013	0.92	1.40	1.28	9.5	0.55	0.96	0.96	0.021	0.020	0.99	0.96	—			17.6
TDS 錠 1 mg/kg	♂	267.5	1.08	0.41	0.015	0.87	1.50	1.23	9.8	0.54	0.84	0.85	0.021	0.021	1.06	1.06	—			17.4
TDS 錠 10 mg/kg	♂	248.7	1.10	0.39	0.012	0.77	1.45	1.23	8.7	0.51	0.76	0.75	0.019	0.020	1.00	1.01	—			16.3
TDS 錠 100 mg/kg	♂	260.8	1.09	0.48	0.015	0.93	1.84	1.42	9.3	0.55	0.90	0.90	0.024	0.022	0.93	0.92	—			16.7

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海軍省 衛生局 衛生課

(昭和 25 年 5 月)

影響を検索した。使用した動物は Wistar 系統系ラットの雄性ラット及び雌性ラットで、飼育条件は上述の通りであるが、雌の場合は産卵をチェックしてから実験を開始した。なお、交付薬物は TDS-N とし、基準交付量の 100 倍、1,000 倍をもつて比較した。

第5図はその結果を示したもので、体重の増加は雌雄とも同一傾向の曲線を示すが、成長率は雌性の方が、やや遅っていた。雌性ラットは対照群と TDS-N 交付群との間に有意の差なく、順調な発育曲線を示した。これを雌性ラットで観察すると、対照群と TDS-N 100 mg/kg 交付群は有意の差なく、TDS-N 1,000 mg/kg 交付群は対照群より僅位の曲線を示して、雌性ラット群と雄性ラット群の中間に位置し、発育良好であった。全体的に観察した場合、これら雌雄ラットの成長曲線は雌雄の差はあるが、一般状態は変わらず、良好な発育を示し、毒性の発現は認められず、且つ雌雄差による特異な反応も認められなかった(第5図)。

なお、雌性ラットの組織は肉眼的に変化なく、病理組織学的精密検査によつても病的変化は認められなかった。第6図は TDS-N 交付ラットの卵巣、子宮の組織の一部を示したもので、対照との間に有意の差異は認められなかった(第6図)。

2) 臓器重量よりみた検討

ラット及びニワトリに TDS-N を連続 90 日間交付して、91 日目に動物を脱血致死せしめ、脳及び内臓諸臓器の重量を計量してそれらの変動の有無を精細に比較検討した。その結果は第3表に示す如くで比較のため TDS 交付群を附記した(第3表)。

a) ラットの場合

ラットにおいては雌性ラット群では大脳、小脳、甲状腺、胸腺、心臓、肝臓、腎臓に差異なく、肺臓はやや減少の傾向を示しているが、極めて僅少で、脾臓、副腎は TDS-N 交付各群間で量の大小に関係なく少差が認められた。前立腺、精嚢は重量少なく精密な計量を行なつて 1/100g の誤差範囲で減少傾向を認めたと過ぎなかつた。但し成長と関連して尾長は TDS-N 交付群の方が長くなっているのが認められた(第3表)。

同様の観察を雌性ラットでも行なつた。即ち、大脳、小脳を除いて TDS-N 500 mg/kg 交付群は全体的に低く、TDS-N 1,000 mg/kg 交付群は対照群と同等かやや上まわる結果を得、全体としては有意の差異は認められなかった。なお、子宮、卵巣も特に認むべき差異はなかつた。また、甲状腺は雌性ラットより大

第4表 TDS-N 経口 90 日間連日経口交付したニワトリの主要臓器重量表 (g)

		体重	卵巣	輸卵管	砂嚢	胃	肝	脾	心	腎		肺	甲状腺	大脳	小脳
										右	左				
対 照	1	2,700	9.0	46.0	30.0	8.0	50.0	1.4	10.4	5.5	6.3	6.7	0.08	2.48	0.46
	2	2,940	9.2	52.0	39.0	8.2	76.0	2.3	9.2	7.4	7.3	9.8	0.12	2.70	0.30
	3	3,300	6.3	58.0	32.0	7.0	78.0	3.2	8.3	7.9	8.7	14.6	0.26	2.50	0.60
	4	3,100	5.6	62.0	36.0	9.3	77.0	3.1	8.3	7.9	10.6	10.5	0.16	2.60	0.30
	5	2,900	5.5	58.0	28.0	7.3	57.0	2.7	8.4	8.1	7.2	12.0	0.12	2.80	0.40
平 均		2,968	7.12	55.2	33.0	7.76	66.4	2.54	8.92	7.36	8.02	10.76	0.15	2.61	0.41
TDS-N 10 mg/kg (経口)	1	2,560	5.9	46.0	32.0	7.3	56.0	2.5	8.2	6.3	7.3	4.7	0.08	2.71	0.40
	2	2,800	5.3	44.0	29.0	6.1	62.0	1.9	8.4	7.7	7.3	10.5	0.20	2.80	0.30
	3	2,787	6.2	54.5	31.2	6.8	57.7	2.3	9.4	7.4	7.2	9.2	0.17	2.33	0.32
	4	3,050	6.4	64.0	30.0	6.8	52.0	2.8	10.3	9.0	10.0	16.0	0.20	1.70	0.30
	5	2,750	7.6	65.0	34.0	7.0	61.0	2.0	10.7	6.6	4.5	5.6	0.20	2.10	0.30
平 均		2,787	6.28	54.7	31.24	6.8	57.74	2.3	9.4	7.4	7.26	9.2	0.17	2.33	0.32
TDS-N 100 mg/kg (経口)	1	3,000	8.9	76.0	37.0	7.3	57.0	3.2	8.4	8.6	9.3	6.0	0.20	3.00	0.30
	2	2,700	6.5	56.0	28.0	7.2	57.0	2.0	8.0	8.6	8.3	14.4	0.30	2.60	0.20
	3	2,640	5.8	62.6	40.0	7.5	58.0	2.6	9.7	8.8	7.0	9.0	0.30	2.93	0.30
	4	2,920	6.6	53.0	29.0	7.6	61.0	2.0	8.1	8.9	7.9	8.3	0.20	2.50	0.40
	5	2,460	7.0	52.0	30.0	7.1	52.0	2.6	8.8	7.5	8.2	10.6	0.18	2.60	0.20
平 均		2,740	6.96	59.92	32.80	7.34	57.0	2.48	8.62	8.48	8.14	9.66	0.24	2.72	0.28

第5表 TDS-N 及び TDS 経口 90日間連続経口
交付した雄性及び雌性ラットの血球数
及び血色素量 (30 例平均値)

ラットの性別		性齢	体重 (g)	血 球 数		血色素量 (%)
				赤血球数 (万)	白血球数	
雄 雌 混交	対 照	8	261	871	7,400	98
TDS-N	経口	8	267	823	8,135	92
100 mg/kg						
TDS-N	経口	8	252	863	7,830	98
1,000 mg/kg						
TDS-N	皮下	8	270	763	8,578	98
10 mg/kg						
TDS-N	皮下	8	278	720	9,327	91
100 mg/kg						
雄 雌 混交	対 照	9	227	748	4,915	95
TDS-N	経口	9	220	792	5,165	100
100 mg/kg						
TDS-N	経口	9	231	824	5,320	99
500 mg/kg						
TDS-N	経口	9	240	727	7,790	98
1,000 mg/kg						
雄 雌 混交	対 照	8	282	809	5,571	90
TDS	経口	8	287	676	8,880	92
1 mg/kg						
TDS	経口	8	248	814	8,610	97
10 mg/kg						
TDS	経口	8	260	904	9,450	93
100 mg/kg						

さい値を示したが、対照と TDS-N 交付群相互間には有意の差は認められなかった (第3表)。

b) ニワトリの場合

ニワトリにおいては体重は対照群に比し、TDS-N 強制経口交付群はやや低い、大脳に小脳を加えた重量は変わらず、甲状腺はやや大、腎臓も TDS-N 交付群がやや対照より大きいが大差なく、胃、脾臓、心臓に有意の差なく、僅かに肝臓において TDS-N 交付群は対照より低い傾向を示した。しかしながら全体的にみた場合は平均しており、異常と思われる所見は認められなかった (第4表)。

4) 血液所見よりの検討

ラット及びニワトリに TDS-N を連続 90 日間交付

第6表 TDS-N 経口 90日間連続経口交付した
ニワトリの血球数及び血色素量

対 照	1	2	3	4	5	平均	赤血球数	白血球数	血色素量
	1	2	3	4	5	平均	400 万	14,080	70 (%)
							380	13,200	63
							360	13,760	64
							300	13,120	67
							310	12,480	60
							350	13,328	65
TDS-N 10 mg/kg (経口)	1	2	3	4	5	平均	320	12,100	62
							400	12,800	70
							390	13,760	68
							400	13,200	72
							380	13,100	64
							374	13,004	67
TDS-N 50 mg/kg (経口)	1	2	3	4	5	平均	350	14,000	63
							315	13,100	60
							400	14,000	68
							450	13,400	76
							380	13,600	62
							374	13,620	66

して、91 日目の血液を採血して、対照と TDS-N 交付群との相互間における変動の有無を比較検討した。その結果は第5表及び第6表に示す如くであつた (第5表、第6表)。

a) ラットの場合

雄性ラットにおいては TDS-N の皮下交付群が赤血球数少なく、白血球数やや多いが、血色素量は変わらなかった。これらの変動はいずれも生理的変動の範囲内であつた (第5表)。しかしながら、これを TDS で比較すると、血色素量は変化なく、白血球数も TDS-N と数及び傾向変わらず、赤血球において大量ではむしろ増加、少量では減少していた。但し、赤血球数において TDS、TDS-N 各交付群は共に対照群における上限、下限内にあり、特異的な変化とは認められなかった。

雌性ラットにおいては赤血球数に有意の変化なく、白血球数は対照群が少なく、TDS-N 交付群がむしろ増加の傾向を示した。このことは血色素量についても認められた (第5表)。

b) ニワトリの場合

ニワトリにおいては赤血球数は対照群では平均 360 万に対し TDS-N 交付群は 374~379 万で僅かに多

く、白血球数は対照群の平均値13,328に対し、TDS-N交付群は13,004~13,630で変らず、血色素量も対照、TDS-N交付群共に66%前後で変動が認められなかった(第6表)。

B. Thiamine 誘導体のニワトリの体重曲線に及ぼす影響並びに産卵に及ぼす影響の検察

ニワトリは成熟後直ちに産卵を開始し、以後一定期間産卵を継続する。従つてこの産卵数を指標とすればニワトリの健康状態を間接的に推察することが出来、これに対する消毒薬物の影響も推察することが可能であることはすでに報告した。同時に卵卵は機械的保護により一定期間を経てヒナとなり、更に一定期間後成熟して産卵を開始し、極めて規則的である上、一定体重のヒナを産出することも容易である。そこでニワトリの孵化から産卵までの成長曲線と産卵の関係を検察し、Thiamine 誘導体のこれに及ぼす影響を比較検討した。

本実験には Thiamine 誘導体の一つ、即ち、TDS-Nを用いたが、TDS-Nの交付実験に先立ち、受精卵より孵化後、直ちに1群を5羽に分ち、毎日一定時刻に体重を測定して成長發育曲線を指標としてニワトリ(ヒナ)の状態を観察した。第7図はこれらニワトリの体重推移曲線を示し、第7表は体重推移表で、各群5羽の平均値である(第7図、第7表)。この推移曲線及び推移表でも明らか如く、ニワトリは逐日的に体重の増加を示し、順調な發育を遂げたが、これらのうち最も体重増加の少ない1群に TDS-N 50mg/kg を、またつぎに体重の少ない1群に TDS-N 10mg/kg を孵化後第115日より各4瓶口交付し、最も体重の多い残りの群を無処置対照として比較観察を行なつた。

その結果、TDS-N 50mg/kg 交付群は交付直後より逐日的に体重増加を示し、体重の増加率も対照群のそれと殆んど同じとなり、第150日目前後には TDS-N 10mg/kg の曲線に近づき、好影響が認められた(第7図、第7表)。

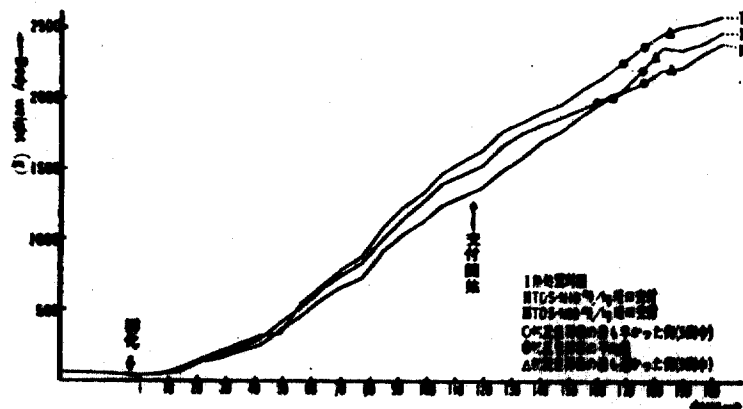
また初産卵日は160日目より200日目の40日間の範囲であり、無処置対照群のニワトリは2,240~2,520g、平均体重2,342gで産卵、TDS-N 10mg/kg 交付群は2,040~2,400g、平均体重2,200gで産卵、TDS-N 50mg/kg 交付群は2,150~2,280g、平均体重2,226gで産卵した。即ち、体重比からこれらと比較すると、TDS-N 交付の各群は大きく、無処置対照群は TDS-N 交付各群より体重100g多くなつてから、産卵を開始した結果となり、TDS-N 交付群の方が対照群より体重が低いにもかかわらず、早い初産卵日をむかえていた。これはさきの体重増加率の促進とともに TDS-N がニワトリに好影響を及ぼしていることを推察させた。なお、第7図のうち曲線上の符号は各群の平均産卵開始日と、各群において最も早く産卵を開始したもの、最も遅く産卵を開始したものに付けて示したものである(第7図)。

C. Thiamine 欠乏症に対する Thiamine 誘導体の効果に関する検察

Thiamine の治療効果を判定するには、Thiamine 欠乏飼料交付により Thiamine 欠乏症をつくり、それに対する効果をもつて判定することは有意義である。

本実験にはマウス、ニワトリ及びハトを使用し、Thiamine 欠乏飼料ないし低栄養飼料として精製白米を各動物に一定期間交付する飼育実験により Thi-

第7図 TDS-N 増量交付ニワトリ(卵卵→孵化→ヒナ→ニワトリ)の成長發育曲線(5例平均値)



第7表 TDS-N 飼量交付エワトリ (鶏卵-孵化-ヒナ-エワトリ) の成長发育体重量 (5例平均値)

時期		飼料日給30g年月日 飼料平均 体重量 5匹		経過日数														
				2/VI	3	4	5	6	7	8	9	10	11	12	13	14	15	16
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
第I期	無処置対照	57.0	57.0	55.8	55.8	55.6	55.0	54.8	54.0	54.0	53.8	53.8	53.0	53.0	52.4	51.4	51.2	
第II期	TDS-N 10 mg/kg (飼口)	57.4	57.4	56.0	55.6	55.0	54.6	54.4	54.2	54.0	54.0	54.0	53.2	52.8	52.0	51.4	51.2	
第III期	TDS-N 50 mg/kg (飼口)	56.8	56.8	55.6	55.2	54.6	54.2	53.8	53.4	53.4	53.4	53.2	52.4	52.4	52.0	51.4	51.2	
時期		飼料日給30g年月日 飼料平均 体重量 5匹		経過日数														
				18	19	20	21	22	23		24	25	26	27	28	29	30	1/VII
		17	18	19	20	21	22		1	2	3	4	5	6	7	8	9	
第I期	無処置対照	51.0	50.0	50.0	49.8	49.4	49.0		38.4	36.0	37.2	42.6	46.2	46.4	47.8	53.2	58.8	
第II期	TDS-N 10 mg/kg (飼口)	51.0	50.2	50.2	49.6	49.4	47.0	北	38.6	36.6	39.4	41.6	47.2	47.8	48.2	50.4	55.4	
第III期	TDS-N 50 mg/kg (飼口)	51.0	50.2	50.2	49.6	49.4	39.4		37.2	35.2	35.0	39.6	41.8	4.22	43.6	48.0	49.2	
時期		飼料日給30g年月日 飼料平均 体重量 5匹		経過日数														
				3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
		10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	
第I期	無処置対照	61.0	65.4	70.8	78.8	86.0	96.0	103.8	110.8	118.8	128.8	137.6	145.0	154.8	162.2	172.0	179.8	
第II期	TDS-N 10 mg/kg (飼口)	58.0	58.6	62.2	65.4	71.2	79.8	82.8	91.2	95.8	105.4	116.0	123.6	132.2	139.2	145.8	152.6	
第III期	TDS-N 50 mg/kg (飼口)	52.2	58.0	59.9	63.0	67.0	76.6	81.8	87.2	93.4	100.8	107.8	114.0	120.2	125.4	132.2	138.2	
時期		飼料日給30g年月日 飼料平均 体重量 5匹		経過日数														
				19	20	21	22	23	24	25	26	27	28	29	30	31	1/VII	2
		26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	
第I期	無処置対照	189.4	196.2	202.2	211.2	219.0	226.4	234.6	241.8	248.6	256.4	260.0	266.2	272.2	279.2	288.6	301.0	
第II期	TDS-N 10 mg/kg (飼口)	160.4	168.8	176.2	189.0	197.0	204.2	212.6	218.8	228.0	236.6	246.2	252.8	258.2	266.2	274.8	284.4	
第III期	TDS-N 50 mg/kg (飼口)	144.8	150.6	155.4	160.6	167.8	173.4	178.8	185.2	191.6	195.8	203.0	208.0	214.0	220.6	232.8	241.2	

鶏卵-孵化-ヒナ-エワトリ

(第25表第5中)

第7表 つづく

試験開始後30年月日		4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19		
時期	飼別平均 体重5匹	経過日数	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	
第I群	無処置対照		311.4	329.8	348.2	371.6	382.8	394.4	509.6	420.0	436.0	450.2	464.6	478.0	493.0	512.4	536.2	554.8	
第II群	TDS-N 10 mg/kg (経口)		294.8	313.2	332	351.6	363.2	375.2	386.0	404.0	418.0	432.4	447.6	463.8	474.0	490.8	506.6	523.3	
第III群	TDS-N 50 mg/kg (経口)		249.8	265.6	282.8	299.0	309.8	321.2	334.6	349.8	365.2	377.2	387.4	400.0	414.2	428.2	445.4	463.8	
試験開始後30年月日		20	21	22	23	26	29	1/IX	4	7	10	13	16	19	22	25	28		
時期	飼別平均 体重5匹	経過日数	58	59	60	61	64	67	70	73	76	79	82	85	88	91	94	97	
第I群	無処置対照		571.2	590.0	600.8	614.0	653.0	724.0	778.0	832.0	864.6	934.0	1008.0	1060.0	1126.0	1204.0	1264.0	1308.0	
第II群	TDS-N 10 mg/kg (経口)		536.0	551.8	562.0	572.0	632.0	694.0	734.0	794.0	822.0	892.0	940.0	986.0	1062.0	1126.0	1202.0	1244.0	
第III群	TDS-N 50 mg/kg (経口)		377.8	483.6	505.8	516.0	562.0	604.0	646.0	692.0	718.0	778.0	836.0	900.0	970.0	1018.0	1074.0	1104.4	
試験開始後30年月日		1/X	4	7	10	13	交付開始		16	17	18	19	20	21	22	23	24	25	
時期	飼別平均 体重5匹	経過日数	100	1003	106	109			112	115	116	117	118	119	120	121	122	123	124
第I群	無処置対照		1364.0	1418.0	1448.0	1492.0			1534.0	1560.0	1568.0	1578.8	1580.0	1608.0	1630.0	1663.6	1694.0	1704.0	1720.8
第II群	TDS-N 10 mg/kg (経口)		1300.0	1358.0	1378.0	1406.8			1435.2	1163.6	1470.4	1479.0	1486.4	1500.0	1516.0	1548.0	1578.0	1592.0	1605.2
第III群	TDS-N 50 mg/kg (経口)		1148.0	1196.0	1232.0	1268.0			1284.8	1305.2	1312.4	1317.6	1324.0	1338.0	1360.0	1381.0	1408.0	1422.4	1440.0
試験開始後30年月日		26	27	28	29	30	31	1/XI	2	3	4	5	6	7	8	9	10		
時期	飼別平均 体重5匹	経過日数	125	126	127	128	129	130	131	132	133	134	135	136	137	138	139	140	
第I群	無処置対照		1733.2	1743.2	1749.6	1758.8	1767.2	1777.2	1786.8	1796.0	1804.0	1813.2	1826.0	1836.4	1849.2	1860.4	1870.8	1880.0	
第II群	TDS-N 10 mg/kg (経口)		1628.0	1640.0	1650.0	1662.0	1674.2	1689.2	1704.8	1718.0	1729.2	1738.4	1745.6	1753.6	1760.4	1770.2	1779.2	1792.0	
第III群	TDS-N 50 mg/kg (経口)		1453.2	1464.0	1478.0	1499.6	1517.6	1526.0	1534.8	1548.8	1564.0	1578.0	1594.0	1608.0	1626.0	1638.0	1654.0	1676.0	

(1967年9月) 施設: サイア: フリマの増産及び繁殖作用に関する実験的調査 第1報

第7表 つづ

試験日数30年月日		11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26		
時期	時期平均 体重5匹	経過日数	141	142	143	144	145	146	147	148	149	150	151	152	153	154	155	156	
第I期	無処置対照		1886.4	1882.8	1886.4	1902.0	1908.0	1916.0	1934.0	1946.0	1964.0	1980.0	1986.0	2014.0	2028.8	2040.0	2052.8	2072.0	
第II期	TDS-N 10 mg/kg (経口)		1798.8	1806.0	1814.4	1821.2	1828.6	1836.0	1844.4	1850.8	1858.8	1870.0	1876.8	1883.2	1889.0	1901.2	1908.2	1918.0	
第III期	TDS-N 50 mg/kg (経口)		1886.8	1894.0	1706.0	1716.4	1721.6	1732.0	1748.0	1766.0	1782.0	1798.2	1810.0	1823.2	1838.4	1852.8	1861.2	1874.0	
試験日数30年月日		27	28	29	30	1/2月	2	3	4	5	6	7	8	9	10	11	12		
時期	時期平均 体重5匹	経過日数	157	158	159	160	161	162	163	164	165	166	167	168	169	170	171	172	
第I期	無処置対照		2080.4	2088.0	2098.0	2110.0	2122.8	2144.0	2170.0	2190.0	2200.0	2210.0	2221.2	2238.4	2282.0	2284.0	2308.0	2310.0	
第II期	TDS-N 10 mg/kg (経口)		1932.4	1944.0	1954.0	1962.4	1971.2	1977.2	1986.0	1994.0	2006.0	2017.2	2038.8	2050.0	2068.0	2082.0	2098.0	2116.0	
第III期	TDS-N 50 mg/kg (経口)		1886.0	1898.8	1916.0	1936.4	1954.4	1968.0	1976.4	1984.4	1995.2	2006.4	2015.2	2024.0	2034.8	2049.6	2064.0	2082.8	
試験日数30年月日		13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28		
時期	時期平均 体重5匹	経過日数	173	174	175	176	177	178	179	180	181	182	183	184	185	186	187	188	
第I期	無処置対照		2318.0	2326.0	2348.0	2356.0	237.2	2393.0	2410.0	2426.0	2434.0	2440.0	2451.2	2454.0	2460.0	2472.0	2478.0	2466.0	
第II期	TDS-N 10 mg/kg (経口)		2124.0	2146.0	2182.0	2214.0	2246.0	2274.0	2292.0	2316.0	2332.0	2344.0	2360.0	2358.0	2360.0	2344.0	2344.0	2330.0	
第III期	TDS-N 50 mg/kg (経口)		2066.8	2077.2	2086.0	2095.2	2108.0	2124.0	2140.0	2161.2	2172.8	2183.2	2196.0	2196.0	2196.0	2196.0	2186.0	2190.0	
試験日数30年月日 試験4年月日		29	30	31	1/I	2	3	4	5	6	7	8	9						
時期	時期平均 体重5匹	経過日数	189	190	191	192	193	194	195	196	197	198	199	200					
第I期	無処置対照		2474.0	2482.0	2490.0	2490.2	2494.0	2498.0	2506.0	2520.0	2530.0	2546.0	2560.0	2554.0					
第II期	TDS-N 10 mg/kg (経口)		2324.0	2336.0	2354.0	2364.0	2372.0	2378.0	2376.0	2384.0	2388.0	2405.8	2414.0	2432.0					
第III期	TDS-N 50 mg/kg (経口)		2202.0	2212.0	2222.0	2232.0	2244.0	2260.0	2270.0	2300.0	2300.0	2310.0	2332.0	2340.0					

amide 誘導体の治療結果に及ぼす影響を検索した。

1) マウスによる検索

12g内外のDD系統雄マウスを用い、マウス飼育用のオリエンタル粉末飼料(以下完全飼料と略記)及び精製白米粉末飼料(以下白米飼料と略記)交付群に大別し、更にその各々をTDS無交付群、TDS 0.5%交付群及びTDS 5.0%交付群に分け、計6群により実験し、比較観察を行なった。この際、TDS無交付の2群は対照群とした。

マウスは毎日一定時刻に1群の合計体重を測定し、30日間持続して観察した。その結果は第8図の如くであつた(第8図)。なお、死亡した場合も平均体重で示さず、合計体重で示したため、飼料差による体重の変化が著しかった。

a) 完全飼料による飼育マウスの場合

完全飼料単独交付による飼育マウスは飼育開始後速目的に体重を増し、多少の増減は認められたが、交付30日まで体重増加の一途をたどり、一般状態は良好であつた。この体重推移曲線を対照とした(第8図の曲線I)。次いで完全飼料にTDSを0.5%添加飼育したマウスは前半の20日間は対照と同一傾向の体重増加を示し、以後徐々に体重の増加率が減少した。しかし一般状態は対照同様良好であつた(第8図の曲線II)。また完全飼料にTDSを5.0%添加飼育したマウスは最初の10日間は対照と同一傾向の体重増加

を示したが、以後体重増加は抑制された。しかし、一般状態は良好であつた(第8図の曲線III)。

b) 白米飼料による飼育マウスの場合

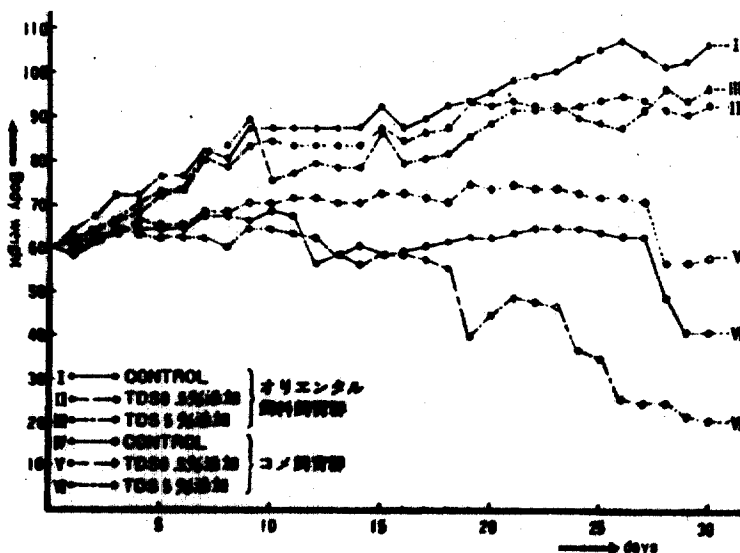
白米飼料単独交付による飼育マウスは飼育開始後間もなく体重増加は著しく抑制され10日前後には死亡する例も認められ、30日に至るも体重は減少のみで、生きのこつたマウスも一般状態は全く不良であつた。この体重曲線をTDS添加の場合の対照とした(第8図の曲線IV)。次に白米飼料にTDS 0.5%添加飼育したマウスは10日前後まで僅かに体重増加を示し、以後急速に体重減少し、28日前後には死亡する例も現われたが、一般状態は対照よりやや良好であつた(第8図の曲線V)。更に白米飼料にTDSを5.0%添加飼育したマウスは20日前後まで僅かな体重増加を認めるが、以後体重は減少し、死亡するものも現われた(第8図の曲線VI)。

c) 完全飼料、白米飼料およびそれらにTDSを添加飼育せるマウス内臓諸臓器の重量及び臓器体重比の検討

マウスに上記各飼料を交付して、31日目に全例を解剖し、臓器の重量及び体重比を測定した結果は第8表及び第9図の通りであつた(第8表、第9図)。

即ち、大脳、小脳、心臓、腎臓、胃には著しい変化なく、肝臓と脾臓とにおいて完全飼料交付の各群と白米飼料交付の各群との間に体重比の差異が認められ

第8図 完全飼料及び白米飼料飼育マウスに対するTDS交付の体重推移曲線



第8表 TDS 配合飼料を連続 30 日間交付したマウス内臓諸臓器の重量 (g) 及び臓器体重指数

	飼料 種類	体重 (g)	A B	大腸	小腸	心臓	肺臓	肝臓	脾臓	腎臓		睾丸	
										右	左	右	左
										有	左	有	左
TDS 配合飼料	無添加	18.8	A	0.31	0.12	0.17	0.17	1.10	0.11	0.15	0.15	0.06	0.06
			B	100	100	100	100	100	100	100	100	100	100
	TDS 0.5% 添加	18.3	A	0.27	0.10	0.14	0.14	0.98	0.08	0.15	0.15	0.03	0.03
			B	150.0	100.0	88.9	87.9	93.1	66.7	115.0	103.6	59.4	62.5
	TDS 50.0% 添加	17.1	A	0.25	0.09	0.14	0.15	0.98	0.07	0.15	0.15	0.05	0.05
			B	147.0	95.0	99.7	101.1	96.3	75.0	112.5	110.8	90.6	93.8
白米飼料	無添加	11.2	A	0.34	0.08	0.15	0.13	0.88	0.06	0.13	0.13	0.08	0.08
			B	218.0	126.7	144.4	131.9	134.5	88.3	146.3	148.2	237.5	243.8
	TDS 0.5% 添加	12.1	A	0.242	0.09	0.16	0.14	1.27	0.57	0.13	0.14	0.08	0.08
			B	119.0	123.3	147.7	127.5	179.3	76.7	140.0	138.6	215.6	218.8
	TDS 5.0% 添加	9.4	A	0.28	0.10	0.13	0.15	0.97	0.07	0.14	0.14	0.08	0.06
			B	296.0	185.0	154.4	168.2	177.6	125.0	186.3	178.3	290.6	293.8

A 平均重量 (5 例の平均値) B 臓器・体重指数

た。

2) エツトリによる検査

平均体重 900 g のエツトリを選び、飼料を白米飼料にきりかえて飼育し、これを 4 群に分け、白米単独交付群を対照とし、他は白米飼料に TDS-N の各々 10 mg/kg、50 mg/kg、100 mg/kg を添加して 30 日間により、体重曲線を指標として比較検査した。その結果は第 9 表、第 10 図の通りであった (第 9 表、第 10 図)。

白米単独交付による対照群は白米飼料交付直後より体重減少著明となり、以後僅かずつ体重を回復し、20 日前後、実験開始時の体重にまで回復し、以後体重は僅かずつ増加を示した。この曲線を対照として TDS-N 交付の各群と比較すると、白米飼料に TDS-N 10 mg/kg 添加群は交付開始後一過性に体重減少を示し、以後徐々に回復に向い 10 日前後で実験開始時の体重にまで回復したのも、漸次体重を増加した。この体重推移は TDS-N 50 mg/kg 添加群の方が増加率で勝っていた。また、TDS-N 100 mg/kg 添加群は TDS-N 50 mg/kg 添加群より体重の増加率が高かった (第 9 表、第 10 図)。しかしながら完全飼料交付の場合と比べると、白米に TDS-N を添加した方は体重増加率極めて低く、成長育曲線は遙かに及ばなかった (第 9 表)。

3) ハトによる検査

体重 300~400 g のハトに白米を飼料として飼育し、

いわゆる Thiamine 欠乏症のハトを作成した。ハトは白米単独交付により、20 日前後で運動減少し、体を丸め、頭を羽にうずめ、ときに振頭を頻発するようになり、30 日前後に至ると、その状態著しくなり、外的刺激による反応にも鈍くなり、40 日を過ぎて、軽度の座学様症状の発現を示すようになった。第 46 日目より、これらのハトに TDS-N の 10 mg/kg、50 mg/kg、100 mg/kg 各量を皮下注射し、また対照として TH の 50 mg/kg、100 mg/kg、200 mg/kg 各量を皮下注射して Thiamine 欠乏症に対する治療効果を比較観察した。

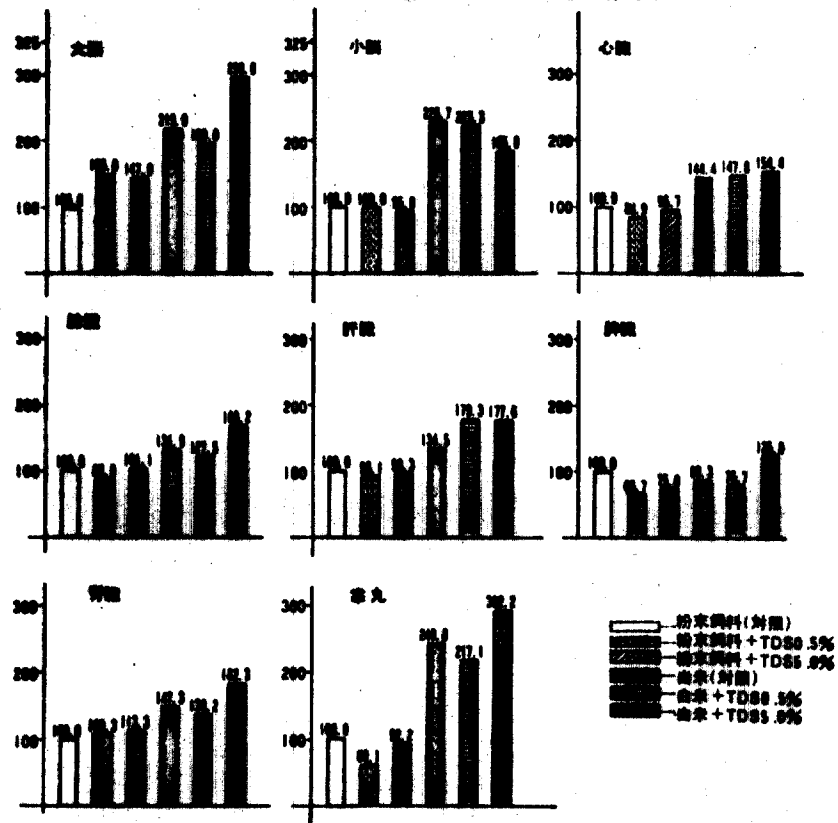
TH は 50 mg/kg で注射後 15 分より振頭ないし座学様症状緩解し、30 分前後には自発運動を回復した。この回復度は 100 mg/kg、200 mg/kg と増量すると一層促進した。TDS-N は 10 mg/kg では効果殆んど現われず、50 mg/kg で注射後 15 分より振頭ないし座学様症状緩解して漸次運動を回復した。また、100 mg/kg では注射後 10 分には振頭ないし座学様症状が消失し、徐々に運動を回復し、pecking などを行なうようになった。TDS-N は TH の効果と同様か、やや優り、Thiamine 欠乏症ハトの治療に効果があることを示した。

IV. 総論並びに考察

以上の実験結果を総括し、次の如く考察する。

1. Thiamine 及びその誘導体の急性毒性をマウ

第9図 完全飼料及び低栄養飼料交付マウスの臓器体重比



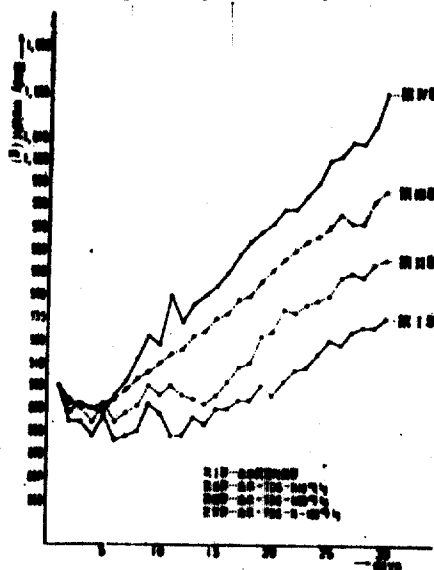
第9表 白米飼料及び白米飼料に TDS-N 量を添加せるニワトリの体重推移表 (30日間) (5例平均値)

日数	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
第I群	900	884	884	878	886	878	878	880	892	888	878	878	886	884	880
第II群	900	884	880	884	892	884	888	892	900	896	900	896	894	892	886
第III群	900	888	888	890	892	884	888	904	906	910	914	916	922	924	930
第IV群	900	888	888	890	888	896	908	912	922	918	940	928	936	940	944
第V群	884	934	1000	1060	1126	1206	1284	1308	1364	1418	1448	1450	1490	1510	1520
日数	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30
第I群	890	884	884	900	886	902	897	908	914	920	918	924	926	926	930
第II群	908	908	910	922	924	934	932	936	938	940	948	950	948	954	956
第III群	932	938	940	946	952	956	960	964	966	970	976	972	984	984	998
第IV群	950	958	964	968	972	978	978	984	990	1000	1004	1008	1008	1016	1016
第V群	1534	1580	1600	1680	1680	1640	1650	1670	1680	1700	1706	1710	1714	1716	1718

第I群: 白米飼育対照 第II群: 白米+TDS-N 10 mg/kg 第III群: 白米+TDS-N 50 mg/kg
 第IV群: 白米+TDS-N 100 mg/kg 第V群: 無塩飼料対照

ス、エワトリ及びハルを用い、比較検討を行なうと、マウスの場合は第12図、第13図の如くである(第12図、第13図)。即ち、TDS及びTDS-NをTHと比較すると、静脈注射ではTDSが5.5倍、TDS-Nが3.1倍となり、腹腔内注射ではTDSが12.8

第10図 曲米飼料及び白米飼料にTDS-N添加量を増加せるエワトリの体重増進曲線(30日間)(5例平均値)



第11図 低栄養飼料飼育による40日ヒナの腹巻発現に対するTDS-Nの効果



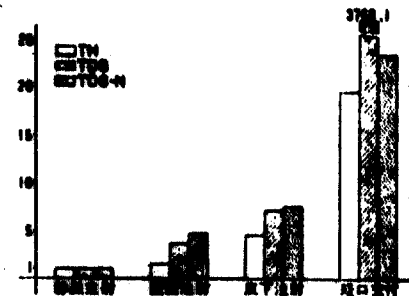
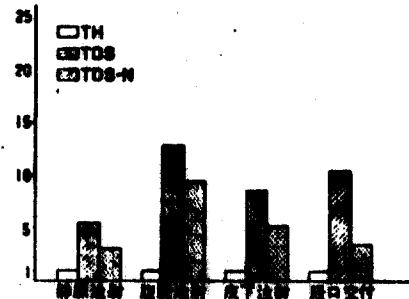
対照(自由給食、40日交付：腹巻発現、自由運動低下)



TDS-N 100 mg/kg 皮下交付 30 分後

倍、TDS-Nが9.5倍となり、皮下ではTDSが8.6倍、TDS-Nが5.3倍となり、経口交付ではTDSが10.6倍、TDS-Nが3.7倍となる。これらの数値が示すように、TDSの急性毒性はTHより著しく弱く、且つTDS-Nの毒性より弱い。TDS-Nの毒性はTHとTDSの中間に位置する。また、TH、TDS及びTDS-Nにつき、交付方法による差異を静脈注射のLD₅₀と比較すると、腹腔ではTH: 1.5倍、TDS: 3.6倍、TDS-N: 4.7倍となり、皮下注射ではTH: 4.7倍、TDS: 7.1倍、TDS-N: 7.7倍となり、経口交付ではTH: 19.6倍、TDS: 37.7倍、TDS-N: 23.1倍となる。即ち、3者ともその傾向は一致しているが、THは何れの交付でもTDS及びTDS-Nより低い数値を示し、毒性は強い。TDSとTDS-N相互間ではその数値は比較的近似であるが、経口交付のみTDSはかなり高い数値を示している。交付部位による上記の差異は吸収と極めて密接な関係にあり、静脈注射は直接血行に、腹腔内注射は腹膜を通して血行に、また皮下注射は組織に入り静脈系を通して吸収されるのに対して、経口では腸からの吸収による。水に易溶性の物質と難溶性の物質とでは吸収に自ら差異のあることは当然考えられるが、第12図に示したごとく急性毒性からもそのことは充分推察出来る。TDSは水に難溶性で、経口の場合毒性が極めて低いのは、この吸収と関連するものと思われる。

またTDSに硝酸を加えて易溶性としたTDS-NはTHより毒性極めて低く、TDSとは異なっている。

第12図 TH, TDS 及び TDS-N LD₅₀ の各々の経口投与をした場合の毒交付方法の差異第13図 TH の LD₅₀ を基準とした場合の TDS 及び TDS-N の LD₅₀ との比較

これらの点からみて、TDS と TDS-N は TH より毒性が低く、TDS-N は TDS より更に腸管からの吸収が優れているように思われる。Zima¹⁰⁾ も TDS が毒性の非常に少ないこと及び効力の持続時間が長い点を報告し、高川¹¹⁾ はマウスにおいては TH と TDS はその効力がほぼ同等であることを認めている。

Thiamine は自然腸においては aneurinase 作用を有する酵素によつて分解され、Thiamine を服用した場合、効力は減退されるといわれ、内服による腸管からの吸収による効力に影響を及ぼす。Thiamine 誘導体、ことに TDS 及び TDS-N は Thiamine における thiazole 部の開環による aneurinase の一つの作用である amine 部と thiazole 部の交換の行なわれることもなく、また他の pyrimidine 部と thiazole 部との切離も行なわれ難い、いわゆる活性型 Thiamine に属するものである。この TDS は Thiamine を alkali 性で酸化することにより、thiazole form の Thiamine 2 分子が結合したもので、その構造中に Thiamine 以外の他の生理作用を異なすような因子の結合はなく、Thiamine 以外の生理的作用を異なす因

子の毒性については全く考える必要はない。したがって TH, TDS, TDS-N の毒性は Thiamine と-S-S-型の disulfide 型 Thiamine 並びにその前駆体の毒と思考される。

マウスにおける急性毒性は上述の通りであるが、ニワトリの場合には TH の 750 mg/kg に対し TDS は 1,075 mg/kg で両者間に 325 mg/kg の差が認められる。即ち、急性毒性は TDS の方が弱い。ニワトリでは一度に多数の卵を得やすく、孵化を行なうことが可能で、飼育方法も極めて容易であるなど、また、幼鳥から成鳥に至る個々の段階を設定出来る利点があり、且つ、Thiamine 系薬物の screening test には極めて有用な実験動物である。このニワトリを 10 日ヒナ、20 日ヒナ、40 日ヒナ、60 日ヒナ及びニワトリ(成鳥)に分けて急性毒性を施行し、各ヒナの成長と毒性の関係及び TH と TDS の相互間における毒性の差は上述した如く TDS が TH より優っている。またハトはニワトリの 10 日ヒナの数値に一致し、且つ、体重からの量的関係の一致をみたことは興味がある。

2. Thiamine の亜急性ないし慢性毒性はマウス、ラット及びニワトリにより行ない、ラットにおいては雌雄差の観察を行ない、長期期間交付における Thiamine 誘導体による影響を詳細に検討を加えた。マウスに 30 日間 TDS を交付した場合、成長半の減少傾向を認めるにとどまり、一般状態も対照と比較して差異が現われない。Zima¹⁰⁾ は同様の実験につき TH と TDS を比較して、TH のみに変化を認め、運動の減少、痙攣の発現、眼球突出及び麻痺の発現を報告している。したがって TH の反復投与は動物を過敏にするが、TDS は毛並みの逆立つ状態を除いて、他に変化なく、TDS の毒性の少ないことを認めている。

ラットにおいては TDS と TDS-N の同一量を比較して有意の差異なく、90 日間に及ぶ一般状態良好で、毒性の発現は認められない。急性毒性で示された如く、TDS と TDS-N は経口交付による LD₅₀ 差は著しい。しかし長期投与からみた場合、両者間に差異はなく、臓器重量、血液所見、さらに病理組織学的所見にも変化がないことは、TDS を前駆体として鼻吸性を高めても毒性が強くないことを推察させるものである。これに関連して、雌雄差は認められず、病理組織上からも変化は認められない。

長期投与からみて、TH は病的症状の発現可能であるが、TDS 及び TDS-N は変化を現わさず、TDS-N は TDS より吸収性において優れているにもかかわらず

ず、毒性は変わらないものと思われ。

3. 鶏卵が孵化し、成長して成鳥となり、産卵を開始するまでを調査し、その間における Thiamine 誘導体の成長並びに産卵に及ぼす影響を調査し、TDS-N は両者に影響を及ぼすことを認めた。殊に初産卵日の到来は無産卵の場合より早く、平均体重が低いにもかかわらず産卵を開始し、一般状態も変らず、血液所見、臓器重量、病理組織学的変化も認められないことから、TDS-N はニワトリの成長発育に好影響を及ぼすものと思われ。

4. Thiamine 誘導体の治療効果に及ぼす影響を Thiamine 欠乏飼育マウス、ニワトリ、ハトを使用して検索し、マウスでは完全飼料交付飼育群、Thiamine 欠乏飼料飼育群、Thiamine 欠乏飼料に TDS を添加した群を区別して観察して、TDS が Thiamine 欠乏マウスに治療効果をあげ、ニワトリ及びハトの Thiamine 欠乏による病的症状発現にも好結果をもたらすことを証明した。この Thiamine 欠乏症は、いわゆる一次的 Thiamine 欠乏症で、絶対的または相対的な Thiamine 欠乏を主とする低栄養である。したがって、二次的な或いは条件付きの Thiamine 欠乏症ではないが、動物においては Wernicke の症候群とかなり類似している。TH は Thiamine 欠乏症に有効であるが、一方では TH は aneurinase 或いは thiaminase により分解されるとされ、川崎、堀尾¹⁰⁾はいわゆる耐熱性因子によつても、Thiamine が分解されることを認めている。しかしながら Thiamine 誘導体のうちには aneurinase の分解を受けにくいものがあり、松井¹¹⁾は TDS は aneurinase の分解を受けないと報告し、村田¹²⁾らは aneurinase 作用を検索して TDS は aneurinase に含まれる還元因子や水解因子により、一旦 Thiamine に変つた上で、はじめて aneurinase 作用を受けるとし、一般に TDS は aneurinase 作用を受けにくいと報告している。また西尾¹³⁾も同様の報告を行っている。

一方、浜本¹⁴⁾らはシロネズミに対する TDS 並びに TH の効果を比較し aneurinase-disulfide による Thiamine 欠乏シロネズミ誘導の面から検索し、TDS と TH の相互間に有意の差異なしとし、高川¹⁵⁾は Thiamine 欠乏シロネズミに対する TDS の効果を長期交付実験により検索して、TDS と TH は効力において著しい差異はないが、体重の面からみて TDS 交付群の方が優れていると報告している。また、Child & Chierini¹⁶⁾は TDS は thiochrom 反応に対し陰

性を示すが、活性型 Thiamine として有効であると述べている。余のマウスにおける検索は上述の実験結果と一致し、ニワトリによる TDS-N、或いはハトによる TH と TDS-N の比較も大体一致をみている。即ち、TDS 或いは TDS-N の腸管よりの吸収が良好で、aneurinase の分解による効力減退を抑制し、藤田¹⁷⁾、Rinde¹⁸⁾、川崎¹⁹⁾らの血中濃度及び臓液への移行についての報告よりみて、また、Marten²⁰⁾の報告にみられる如く動物組織中の TDS の存在、Peterlin²¹⁾の肝 co-carboxylase 測定値よりみた組織内沈着、また Rosanov²²⁾による腸口交付による腸管吸収などからみて、体内貯留性と持続性、或いは組織への親和性がよく生体内で co-carboxylase になり易く、極めて安定性に優れていると思われ。

V. 結 論

以上の実験結果を次の如く結論する。

1. サイアミン (Thiamine) 及びその誘導体は急性毒性の検索で、Thiamine (TH) 誘導体の Thiamine disulfide (TDS) 及び Thiamine disulfide nitrate (TDS-N) の方が著しく毒性が低下する。TDS が腸口交付の場合、毒性が殊に低位にあるのは主に吸収が悪いことによると推測すべきである。

2. サイアミン及びその誘導体は一定量を交付するとき諸種動物において、やや長期交付実験において、何れも殆んど毒性を現わさない。TDS、TDS-N の方が毒性は著しく低下し、殆んど皆無である。動物の健康の危はない。

3. サイアミン及びその誘導体はラット、ニワトリの低栄養飼育の場合、成長発育を促進させる効果を示す。

4. ニワトリはヒナ及び成熟のニワトリとともにサイアミンの効力実験では最も適当の実験動物であるが、その孵化成熟から産卵までの過程において、TDS の交付によつて産卵日の到来を早める傾向がある。

5. 諸種の動物において、TDS と TDS-N の作用は相互間に有意の差異は認めない。血液所見及び病理組織学的検索においても同様である。TDS-N の水溶性である点は使用上の便宜を保有している。

以上要するにサイアミン誘導体 TDS 及び TDS-N は毒性低く、動物の成長発育に悪影響を及ぼさず、治療的効力を保有すると認める。

本研究に關して終始御懇篤な御指導並びに御校閲を賜つた恩師原三郎教授に対し、深甚な謝意を捧げる。

なお、本論文の要旨の一部は第 35 回日本薬理学会関東部会で報告したものである。

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Effect of High Intakes of Thiamine, Riboflavin and Pyridoxine on Reproduction in Rats and Vitamin Requirements of the Offspring

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ABSTRACT Female rats were fed during pregnancy and lactation a control diet containing 0.25 mg pyridoxine, 0.4 mg thiamine, and 0.4 mg riboflavin/100 g, or a high pyridoxine, high thiamine, or high riboflavin diet containing the specified vitamins at 25 times the level of the control diet. The levels of these vitamins in the carcasses of the young at birth and in the liver at weaning were determined. The effect of maternal vitamin intake on the vitamin requirements of the young was tested in 2 ways: 1) by comparing the rate of depletion of the young of females fed the high vitamin diets with the rate of depletion of the control group, and 2) by the growth response of the depleted young to graded levels of the vitamin fed in excess in the maternal diet. It was concluded that high intakes of thiamine, riboflavin, or pyridoxine during the reproductive period had no effect on the young, as shown by litter size at birth, growth until weaning, or their vitamin requirements after weaning.

Relatively little attention has been given to the effects of high intakes of B vitamins on reproduction. Massive doses of thiamine have been reported to interfere with lactation, produce cannibalism, and decrease fertility in rats (1, 2). Richards (3) also reported that excess thiamine fed to female rats increased mortality and decreased the weights of the young at weaning. However, Morrison and Sarett (4) reported normal reproduction in rats fed high levels of thiamine. An abnormally high need for pyridoxine was reported in a newborn human infant whose mother had received large doses of pyridoxine intramuscularly during the first trimester of pregnancy (5). It was suggested that excess pyridoxine intake during gestation may have increased the pyridoxine requirement of the infant. Later, Hunt (6) reported that the pyridoxine intake of the dam during gestation did not affect the rate of vitamin B₆ depletion or increase the occurrence of convulsions in young rats after birth. Morrison and Sarett (4) also reported that a high intake of pyridoxine during pregnancy did not affect reproduction or increase the rate of vitamin B₆ depletion in young rats in the period from 3 to 5 weeks of age.

The preceding studies have shown that high levels of pyridoxine in the maternal

diet did not appear to increase the vitamin B₆ requirement of young rats as indicated by rate of vitamin B₆ depletion. If this had been the case, then vitamin B₆ deficiency should have developed more rapidly in the young from the dams fed the high pyridoxine diets. No test was made, however, of the pyridoxine requirement of the young rats after they had been depleted of pyridoxine. This point is important, since an increased need for pyridoxine in the young during the depletion period may have been masked by the higher liver stores of the young from the dams fed the high pyridoxine diets. Greater liver stores were reported by Morrison and Sarett (4).

The following experiment tested the effect of maternal intake of pyridoxine, thiamine, or riboflavin on the rate at which the young rats were depleted of each vitamin, as well as on the subsequent growth response of the depleted young to graded levels of the vitamins.

METHODS

Animals. Thirty female rats (about 300 g) of the Long-Evans strain, were used for each of 3 experiments. In each experi-

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ment, 15 females were fed the basal diet.¹ The other rats were divided into 3 groups of 5 rats each. One group was fed the high pyridoxine diet, the second group the high thiamine diet, and the third group the high riboflavin diet. The high vitamin diets contained 25 times the level of pyridoxine, thiamine, or riboflavin used in the basal diet. The basal diet contained: (in mg/100 g) pyridoxine, 0.25; thiamine, 0.4; and riboflavin, 0.4 (7). The high vitamin diets contained: (in mg/100 g) pyridoxine, 6.25; thiamine, 10; and riboflavin, 10. Food and water were provided ad libitum. Weight gain and food intake were recorded 3 times weekly.

The females were mated after they had been fed the experimental diets for 2 weeks, and these diets were continued during gestation and lactation. The number and average weight of the newborn were recorded as soon as possible after the birth of each litter. Some of the newborn from each diet group were decapitated, the stomachs and intestinal tracts removed, and the carcasses weighed and frozen. The remaining young were nursed for 21 days, when they were weaned. The young had access to the maternal diet during the nursing period. At weaning, representative young from each group were decapitated, the livers removed, weighed and frozen. A number of the male weanlings, weighing from 40 to 50 g, were saved for the growth study.

The growth study was divided into a depletion period and a supplementation period. The rats were housed individually in galvanized, screen-bottom cages. Food and tap water were given ad libitum. Weight gains and food intakes were recorded 3 times weekly. Four to five animals from the high vitamin maternal groups were fed the basal control diet, with the omission of the vitamin which had been fed in excess in the maternal diet. Weanlings from the dams fed the basal diet were also fed the vitamin-deficient diets. The remaining rats from each maternal group served as controls and were fed the complete basal ration. All of the diets were fed until the deficient groups ceased to gain (weight plateau) or until their rate of gain was significantly less than that of the appropriate control group.

Once depletion was established, the animals were fed the appropriate vitamin at a below-minimal or minimal level. The minimal level for pyridoxine was 15 μ g/rat/day (4);² for thiamine, 10 μ g/rat/day (8); for riboflavin, 24 μ g/rat/day (7). The below-minimal levels were one-half of the minimal level. Negative controls (without pyridoxine, thiamine, or riboflavin) also continued to be fed the deficient diets.

Each level of the vitamin was made up in a 20% ethyl alcohol solution. Two milliliters of this solution were fed 3 times a week. The total supplement over a period of one week was equivalent to 7 times the daily amount indicated.

Vitamin analyses. The carcasses of the newborn and the livers of the weanlings were homogenized and analyzed for pyridoxine, thiamine, and riboflavin. Pyridoxine was determined microbiologically with *Saccharomyces carlsbergensis* in a modification of the method of Rabinowitz and Snell (9). Thiamine was determined by the thiochrome method and riboflavin was determined fluorometrically (10).

RESULTS

Reproduction. Reproduction data are shown in table 1. The reproduction of the control group and the high thiamine group was considerably better (68% for both groups) than that of the high riboflavin or high pyridoxine groups (38 and 47%, respectively). The value of 68% was slightly less than that usually found for the stock colony females of this laboratory (70 to 75%).

The average birth weight, number of young per litter, and average weight of the young at weaning were not significant.

¹ Composition of basal diet: (g/100 g) vitamin-free casein, 18; sucrose, 61.8; USP Salts, 14.4; Cellulose (Chicago Dietetic Supply House, Chicago), 5; cottonseed oil, 9; vitamin-fortified cottonseed oil, 1; vitamin mix in sucrose, 1; choline chloride, 0.15. The fortification provided per 100 g diet: vitamin A, 1700 IU; vitamin D, 100 IU; α -tocopheryl acetate, 6.7 mg; thiamine, 0.4; riboflavin, 0.4; pyridoxine, 0.25; pantothenate, 2.0; inositol, 10.0; biotin, 0.01; nicotinic acid, 0.1; niacinamide, 1.0; vitamin B₁₂, 0.1 μ g; folic acid in mannitol, 0.02; menadione, 0.5.

² The minimal requirement for pyridoxine for maximal growth in the rat beyond 6 weeks of age has recently been shown to be greater than 30 μ g/day (Boston, G. H., and M. C. Cheney, Federation Proc. 24: 624, 1965; Williams, M. A., Federation Proc. 24: 624, 1965). However 15 μ g/day will produce nearly maximal growth in the rat for the first 2 weeks after weaning or the first 2 weeks of repletion of previously depleted rats (Williams, M. A., unpublished results).

TABLE 1

Reproduction data on animals receiving a diet containing levels of thiamine, riboflavin or pyridoxine 25 times that of the control level¹

Maternal diet	No. of mothers	No. of litters	No. of young	Average birth wt	Survival	Average wt at 21 days
Control ¹	40	27 ² (68%)	235	6.2	78.6 (214) ³	46.2
+ thiamine (10 mg/100 g diet)	19	13 (68%)	105	6.6	89.4 (98)	48.8
+ riboflavin (10 mg/100 g diet)	13	5 (38%)	51	6.2	60.0 ⁴ (40)	47.9
+ pyridoxine 6.25 mg/100 g diet)	15	7 (47%)	64	6.1	83.0 (53)	45.8

¹ Control diet contained: (mg/100 g diet) thiamine, 0.4; riboflavin, 0.4; pyridoxine, 0.25.

² Cumulative figure; each experiment included a control group of approximately 10 animals.

³ Numbers in parentheses refer to number of observations on which percentage is based.

⁴ Ten offspring that did not survive in this group were all of one litter; therefore the apparent difference loses significance.

TABLE 2

Effect of the level of thiamine, riboflavin or pyridoxine in the maternal diet on the storage of the vitamin in the fetal carcass and in liver of offspring at weaning

Maternal diet	Fetal carcass storage			Liver storage		
	Thiamine	Riboflavin	Pyridoxine	Thiamine	Riboflavin	Pyridoxine
	μg/g	μg/g	μg/g	μg/g	μg/g	μg/g
Control ^{1,2}	0.93 ± 0.27 ³ (5) ⁴	3.43 ± 0.28 (8)	0.79 ± 0.15 (7)	5.81 ± 0.81 (8)	21.48 ± 2.53 (5)	7.43 ± 1.94 (10)
+ thiamine (10 mg/100 g diet)	0.95 ± 0.11 (5)	—	—	10.13 ± 0.75 ³ (8)	—	—
+ riboflavin (10 mg/100 g diet)	—	3.26 ± 0.19 (10)	—	—	22.41 ± 3.64 (5)	—
+ pyridoxine (6.25 mg/100 g diet)	—	—	1.23 ± 0.11 ³ (6)	—	—	7.12 ± 0.82 (10)

¹ Control diet contained: (mg/100 g diet) thiamine, 0.4; riboflavin, 0.4; pyridoxine, 0.25.

² Each vitamin analysis had its own control group.

³ Mean ± s.e.

⁴ Numbers in parentheses indicate number of samples analyzed.

⁵ Difference is statistically significant ($P < 0.01$).

cantly influenced by increasing the levels of pyridoxine, thiamine, or riboflavin in the maternal diet. The number of offspring per litter from the dams fed the high thiamine ration was somewhat smaller than that from the other maternal groups. The average birth weight, however, was somewhat greater in the high thiamine group. The higher mortality (birth to weaning) in the high riboflavin group reflected the complete loss of one litter (10 young). Two control litters were also lost because the mothers failed to nurse. More data are needed to determine whether the high riboflavin diet increased mortality.

Tissue vitamin storage. High levels of thiamine or riboflavin in the maternal diet did not increase the levels of vitamins in the newborn (table 2). The level of pyridoxine in the newborn from the dams fed the high pyridoxine diet was significantly higher than the level for the control group ($P < 0.01$). At weaning, the offspring of the females fed the high thiamine diet had nearly twice as high a level of liver thiamine as did the controls. In contrast with the effect of thiamine, high levels of riboflavin or pyridoxine in the maternal diet did not influence the storage of these 2 vitamins in the livers of the weanlings. The

difference caused by maternal diet in the tissue concentration of pyridoxine at birth did not appear at weaning (21 days after birth).

Growth studies — depletion and supplementation. Figure 1 shows the growth curves of the different groups when fed diets deficient in pyridoxine, thiamine, or riboflavin, as well as the control groups. The only difference observed during depletion was that the onset of thiamine deficiency was delayed slightly in the rats from the dams fed the high thiamine diet. Maternal diet did not affect the development of riboflavin deficiency, in which both groups reached a plateau in body weight by day 21. The depletion period of vitamin B₆ was arbitrarily ended on day 28 when the weight gains of the deficient groups were significantly less than the gains of the groups fed the control diet. Maternal pyridoxine intake did not affect pyridoxine depletion in the young (fig. 1). Neither did the maternal diet affect the growth response of any of the groups to the control diet.

Figure 2 shows the growth response of the vitamin-depleted rats to graded levels of the corresponding vitamin. All groups received the supplements for 14 days. Growth reflected the level of supplementa-

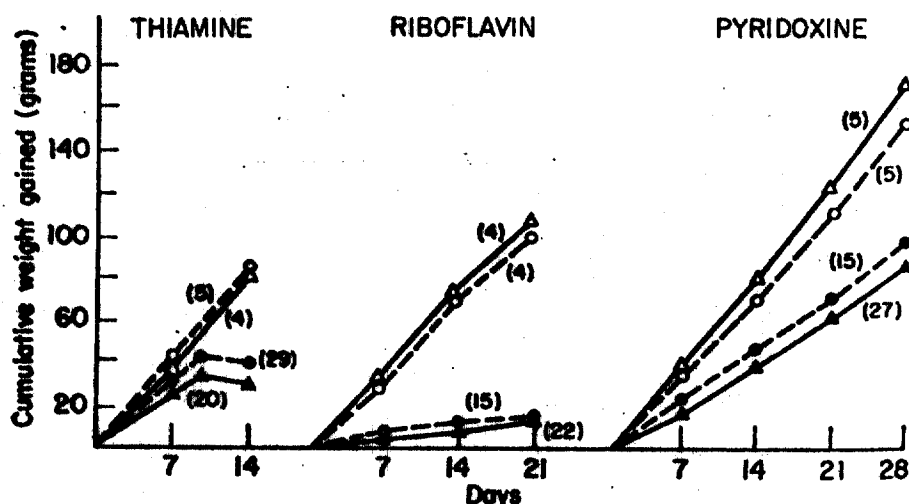


Fig. 1 Influence of excess thiamine, riboflavin, or pyridoxine in maternal diet on weight gain of male weanling rats fed a diet deficient in the respective vitamin. Δ—Δ, control young fed adequate diet; O---O, experimental young fed adequate diet; Δ—Δ, control young fed depletion diet; O---O, experimental young fed depletion diet. Numbers in parentheses indicate number of rats in the group.

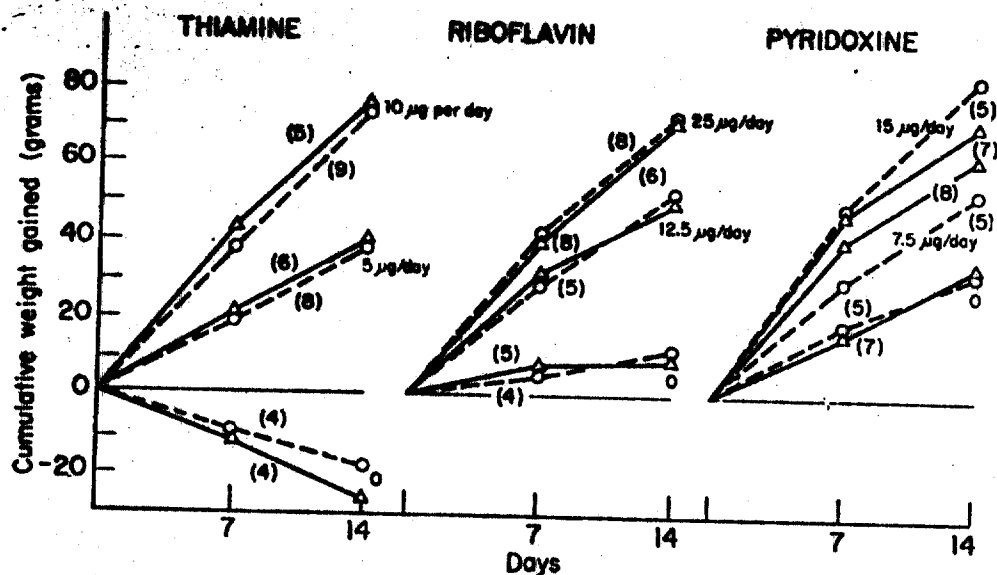


Fig. 2 Influence of excess thiamine, riboflavin, or pyridoxine in maternal diet on weight gain of previously depleted male rats fed graded levels of that vitamin. Δ — Δ , control young; \circ — \circ , experimental young. Numbers in parentheses indicate number of rats in the group.

tion for each vitamin, and maternal diet had no significant effect. The greater variability in the growth of both pyridoxine-depleted groups to supplements of 7.5 or 15 µg pyridoxine is typical of the response of pyridoxine-depleted rats fed at these levels of the vitamin.⁴ Nevertheless, the rats from the dams fed the high pyridoxine diet made weight gains similar to those from the control dams. If the need for pyridoxine had been greater in the young from the high pyridoxine dams, then their weight gains at both levels of pyridoxine would have been less than those of the control young. This lack of a difference in pyridoxine need between the young from the high pyridoxine dams and the control group is supported by the similar weight changes in the pyridoxine-depleted groups that continued to be fed the pyridoxine-deficient diet.

DISCUSSION

The reduced number of litters for the high riboflavin and high pyridoxine groups may be related to the high maternal intake of those vitamins. More data are needed to establish this point. With respect to the number of young per litter and the average birth and weaning

weights, all of the high vitamin groups and the control group compared favorably in performance with the laboratory breeding colony. The results from the high thiamine diet confirm the conclusions of Morrison and Saret (4) that high intakes of thiamine did not affect the overall reproductive performance, in contrast with the report of Richards (3).

The level of thiamine (0.93 µg/g) in the tissues of the newborn from either the control or the high thiamine group was less than that reported by Barrett and Everson (11) and by Brown and Snodgrass (12). Barrett and Everson reported a value of 3.0 µg/g with a maternal diet containing 1.32 mg/100 g. Brown and Snodgrass reported a level of approximately 2 µg/g with a maternal diet containing 0.25 or 0.50 mg/100 g. There is no explanation for these differences except that the analyses were not made on comparable tissues. In the present study, the vitamin analyses were made on the exsanguinated carcass, with the head, stomach, and intestinal tract removed. The other reports imply that the analyses were made on the entire animal. Fetal storage of riboflavin (3.34 µg/g) was similar to

⁴ Williams, M. A., unpublished results.

the values of Barrett and Everson (2.5 $\mu\text{g/g}$). No values for fetal storage of pyridoxine were found in the literature.

At weaning, liver storage of thiamine reflected the thiamine level of the maternal diet although the maternal diet did not affect the carcass thiamine of the newborn. The higher level at weaning may reflect the amount of maternal diet eaten by the young before weaning. The level in the control weanlings (5.8 $\mu\text{g/g}$) is higher than the value (1.9 $\mu\text{g/g}$) observed by Morrison and Sarett whose control diet contained only 0.15 μg thiamine/100 g, in contrast with 0.4 mg/100 g in the present study. Ochoa and Peters (13) and Byerrum and Flokstra (14) had reported previously that increasing the dietary thiamine above that needed for maximal growth increased the tissue concentrations of thiamine.

High levels of riboflavin or pyridoxine in the maternal diet did not influence the liver storage of these 2 vitamins at weaning. The value for riboflavin (21.5 $\mu\text{g/g}$) was similar to the value observed by Decker and Byerrum (15) in rats 30 days after birth (24.5 $\mu\text{g/g}$). The similarity of values for liver pyridoxine in both the high pyridoxine and the control groups differs from the results of Morrison and Sarett, who observed increased levels of pyridoxine in the livers of weanlings from dams fed high pyridoxine diets (7.5 mg/100 g) in contrast with a control diet containing 0.15 mg/100 g. Their values were 8.8 $\mu\text{g/g}$ for the high pyridoxine group and 4.8 $\mu\text{g/g}$ for the control group. In the present paper the values were 7.1 $\mu\text{g/g}$ for the high pyridoxine group and 7.4 $\mu\text{g/g}$ for the control. Perhaps the level of pyridoxine in the diet of Morrison and Sarett (4) was too low for maximal tissue storage, although this is above the stated requirement (16).

The growth studies gave no evidence for any beneficial effect in the young resulting from high maternal intakes of these vitamins during pregnancy and lactation. In addition, there was no evidence that the vitamin requirements of the offspring were increased, that is, the rats were made more vitamin-dependent, as a result of high maternal intakes. The slight delay in the development of thiamine deficiency in the

rats from the high thiamine maternal diet probably reflects the increased tissue thiamine storage at weaning.

In conclusion, high levels of thiamine, riboflavin, or pyridoxine ingested during the reproductive period had no effect on the young, as shown by litter size at birth, growth until weaning, or vitamin requirements after weaning.

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VITAMIN B COMPLEX AS A DETERRENT TO SKIN IRRITATION.

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THE object of the experiments reported in this paper was to find out whether rats could be made more resistant to a skin irritant by the addition of Vitamin B complex to their normal diet. Previous experiments had shown that rats were made much less susceptible to grafts of sarcoma when Vitamin B was added to their diet of hospital scraps (Scott, 1949).

The rats used for these experiments were all of a black and white breed, which for many generations has been fed on a diet of hospital scraps consisting of meat, fish, puddings, milk, cheese and other fats, fruit, vegetables—both raw and cooked—a large quantity of vegetable peelings and dry bread. For many years the rats have kept in excellent condition on this diet and have been singularly free from disease.

A 0.5 per cent solution of 9,10-dimethyl-1,2-benzanthracene in benzene has been used, as this has been shown by many to produce definite skin lesions. Berenblum (1949) has given the comparative results of treating the skin of the mouse, rabbit, rat and guinea-pig.

EXPERIMENTAL METHOD.

Young rats from 70-150 g. in weight were used, great care being taken that controls and experimental rats should be of the same sex, age and weight; when possible a litter was equally divided into control and experimental rats.

A yeast extract and a synthetic Vitamin B mixture were supplied by Mr. F. A. Robinson (of Messrs. Allen and Hanbury) who made it possible for the feeding experiments to be carried out on a quantitative basis. The yeast extract was made by aqueous extraction of food yeast (*Torula*) and concentration of the extract to about one-tenth of its original weight.

The Synthetic Vitamin B mixture consisted of:

Anserine Hydrochloride	150 mg.
Riboflavin	100 "
Pyridoxine	100 "
Pantothenic acid	500 "
Nicotin	5 "
Folic acid	5 "
Lactose	85 g.

The addition of lactose made it easier to weigh and distribute evenly to the animals; 150 mg. of this mixture was estimated as equivalent in vitamin content to 2½ g. of yeast extract. Both mixtures were freshly mixed in water every day and a measured quantity added to the attractive bits in the rats' food, which

were certain to be eaten. The controls had similar scraps with no additions. In all cases, once begun, the extra feeding was kept up for the rest of the rat's life.

A square centimetre was closely clipped in the middle of the rat's back and two drops of the solution (approximately one-twentieth c.c.) dropped on the bald area. The close clipping with scissors gave much the same result as shaving, but avoided the risk of damaging the skin with a razor.

Notes were made on the exact condition of the skin every week; when spots and warts appeared measurements were made with dividers on a mm. scale.

Experiments.

Group A.—All the experimental animals in this group had a total of 2½ g. of yeast extract per week added to their scrap diet. The controls were from the same litters, matched in sex with the experimental rats.

Once a week two drops of dimethyl-benzanthracene were dropped on a closely clipped area 1 cm. square in the middle of the back.

There were eight different experiments (84 rats); the length of time of special feeding before the treatment began varied from 3½ to 9 weeks. The treatment lasted 3½, 4, 4½, 5 or 5½ months.

In due course the usual effects appeared—rough skin, small spots, then warts and eventually, in many cases, definite tumours. Table I shows the onset of the first definite spots and the area covered by lesions on the last date before any of the rats had to be killed. The number of rats which developed definite tumours is also given.

It will be seen from Table I that the yeast feeding appears to have delayed the onset of lesions; the area covered by the warts was also only over half the affected area in the controls (0.54 of the control area) and the percentage which developed definite tumours was 73 against 77 in the controls. All the controls developed definite skin lesions, but 3 of the experimental rats remained free.

The rate of growth of the warts in the experimental rats was slower on the whole than the controls.

Group B.—Thirty-two rats in this group of experiments were given three times the weekly dose of 2½ g. of yeast extract used in Group A. The parents too were fed on this diet before they mated.

Thirty-four rats of the same age and sex were taken from the ordinary stock for controls, the finest specimens being chosen, as the yeast feeding generally adds about 30 or 30 per cent to a young rat's weight, and they compare favourably with the finest specimens in the stock litters.

The length of treatment with dimethyl-benzanthracene varied from 3 to 6 months. As will be seen from Table I, the resistance was considerably increased by the larger dose of yeast in which the parents shared. The percentage of definite tumours was 25 in the experimental rats against 65 in the controls, a great improvement on Group A, and the area covered by warts in the experimental rats was only a third of that in the controls. All the controls developed definite lesions, but two of the experimental rats remained free all their lives.

Group C.—All the rats in this group were treated with dimethyl-benzanthracene in the same way as Groups A and B. The treatment lasted 5 or 6 months. The 22 experimental rats were given a dose of the synthetic B mixture calculated as equivalent to twice the dose of yeast extract in Group A: previous experiments

	Total number of rats.		Months after treatment began.										Rats with no lesions.	Cumulative percent.*	Rats with definite tumours.
			4.	5.	6.	7.	8.	10.	12.	14.	16.	20			
Experimental Group A (10 g. yeast extract per week)	44	Percentage which developed skin lesions.	14	25	66	75	84	91	93	93	93	93	2	Exp. 0-54 of control area	73
		Average area of lesions in sq. mm.	—	—	—	83	196	368 (36)	325 (26)	— (30)	— (14)	— (4)			
Controls (no special feeding)	60	Percentage which developed skin lesions.	30	66	80	83	90	96	96	96	96	100	0	—	77
		Average area of lesions in sq. mm.	—	—	—	150	253	540 (36)	545 (26)	— (15)	— (12)	— (2)			
Experimental Group B (10 g. yeast extract per week)	32	Percentage which developed skin lesions.	3	28	44	63	66	75	78	91	94	94	3	Exp. 0-3 of control area	25
		Average area of lesions in sq. mm.	—	—	4	21	47	126	140 (27)	310 (23)	210 (16)	—			
Controls (no special feeding)	34	Percentage which developed skin lesions.	15	29	50	74	77	86	94	94	100	100	0	—	65
		Average area of lesions in sq. mm.	—	—	10	124	93 (23)	254 (33)	478 (30)	800 (24)	542 (14)	—			
Experimental Group C (Synthetic B mixture = dose A x 2)	23	Percentage which developed skin lesions.	26	44	48	100	—	—	—	—	—	—	0	Exp. 0-54 of control area.	86.5
		Average area of lesions in sq. mm.	—	—	—	80	170	620 (19)	920 (10)	1,870 (1)	—	—			
Controls (no special feeding)	22	Percentage which developed skin lesions.	27	63	95	100	—	—	—	—	—	—	0	—	91
		Average area of lesions in sq. mm.	—	—	—	300	270 (21)	735 (17)	700 (5)	1,510 (2)	2,030 (2)	—			
Experimental Group D (1 mg. aneurine per week)	13	Percentage which developed skin lesions.	0	0	8	15	31	31	54	69	69	69	6	Exp. 0-2 of control area.	0
		Average area of lesions in sq. mm.	—	—	—	—	—	13	35	—	—	—			
Controls (no special feeding)	18	Percentage which developed skin lesions.	11	11	39	39	50	61	89	—	—	—	0	—	44.5
		Average area of lesions in sq. mm.	—	—	—	—	—	26	125	165 (14)	450 (14)	59 (6)			

The numbers in brackets underneath the area figures denote the number of rats still alive. After many rats have had to be killed because of their large tumours the average areas have not been filled in as comparison between the experimental and controls then often becomes misleading.

* The comparative areas in this column are taken from the measurements made on the last day before any of the rats had to be killed because of their skin condition.

with tumour grafts had shown that the synthetic B mixture was not as powerful as the yeast extract. The parents, too, were fed on this mixture.

The results were very similar to those in Group A although the dose was calculated to be twice as much. The onset of the warts was less delayed than in Group A, but the area covered by lesions in the experimental rats on the last day before any were killed (0.54 of the control area) and the percentage of definite tumours was 86.5 in the experimental group against 91 in the controls.

Group D.—Fourteen rats were given $\frac{1}{2}$ mg. aneurine hydrochloride per week; 7 had treatment with dimethyl-benzanthracene for 3 months and were fed on aneurine hydrochloride for 3 weeks before beginning treatment; the other 7, the offspring of aneurine hydrochloride-fed parents, had treatment for 5 months. There were 18 controls. The results of the two experiments are given together, as there was little difference between the rats fed for a shorter time before beginning the short treatment and those treated for a longer time, which were the offspring of parents on the same diet. A very marked protection was shown by the aneurine-fed rats, none of them developed definite tumours against 44.5 per cent of the controls; 6 of the 14 aneurine-fed rats had no lesions, whilst only 1 of the controls remained free.

DISCUSSION OF RESULTS

From these experiments it appears to be possible to increase a rat's resistance to the ill effects of several weeks' treatment of the skin with a 0.5 per cent solution of 9,10-dimethyl 1,2-benzanthracene, by adding Vitamin B to its diet of hospital scraps. The effect of the yeast extract was much more marked when the dose was increased and the parents, too, were fed on the extra vitamin. A dose of $2\frac{1}{2}$ g. per week added to the scrap diet produced a definite effect, the onset of warts was delayed and they did not grow so quickly, the area covered by the lesions was only just over half that in the controls on the last day before any rat had to be killed. When the dose of yeast was increased to $7\frac{1}{2}$ g. a week and the parents, too, shared this diet, the results were much better, the area of the lesions in the experimental rats was only a third of the area in the controls and the percentage of rats which eventually developed definite tumours was 25 against 65 in the controls. In both groups all the control rats developed lesions, while 2 or 3 of the experimental rats remained free in each group.

In Group B it is doubtful how much the rats really consumed, as $7\frac{1}{2}$ g. of yeast extract per week makes the food very bitter and a fraction was generally left.

The synthetic B mixture given to a group of rats (C) gave much the same result as the yeast extract in Group A, although the dose was estimated as equivalent in Vitamin B content to twice as much as the yeast extract, and the rats were the offspring of parents on the same synthetic B diet.

Rats fed on $\frac{1}{2}$ mg. of aneurine a week which were the offspring of parents on the same diet showed a marked resistance to the 9,10-dimethyl 1,2-benzanthracene. None of the 14 rats developed definite tumours, while 44.5 per cent of the controls did: 6 of the aneurine rats remained free of any skin lesions, while only 1 of the controls escaped.

The length of life was prolonged beyond the normal by the addition of Vitamin B to the rats' food. Many of these lived to be as much as two years old and were in quite good condition at that age.

Table II shows the survival figures. In the case of the synthetic B fed rats, the numbers alive at 1 year instead of 2 years is given, as only 2 of the rats in this group lived to be as much as 18 months old.

TABLE II.—*Effect of Addition of Vitamin B to Diet on Length of Life of Rats.*

Feeding.	Number of rats alive when 2 years old.		%
2½ g. yeast extract	7 out of 44		16
Controls	2 „ 40		5
7½ g. yeast extract	8 „ 32		25
Controls	1 „ 34		3
½ mg. Aneurine	7 „ 13		54
Controls	3 „ 18		17
Synthetic B mixture	12 „ 22		55
Controls	5 „ 22		23

} at 1 year.

The rats do not dislike aneurine or the synthetic mixture. Probably better results could be obtained by using a small dose of yeast extract with a much larger dose of aneurine than that given here. The Tables do not show the gradual changes from spots to warts to definite tumours, but when the results are studied in detail the difference between the experimental rats and the controls is rather more marked than the tables show.

The difference in the rate of onset of the warts in the different groups of controls is probably due to either the length of treatment with dimethyl-benzanthracene, or the age of the rat when treatment began, or the variation in the toxicity of the different consignments of dimethyl-benzanthracene. None of these differences affects the comparison of the experimental rats with their own controls, as these variables were kept constant in each experiment.

SUMMARY OF RESULTS

When yeast extract or synthetic Vitamin B mixture or aneurine hydrochloride is added in the diet of a rat its skin can become resistant to the irritating effects of a 0.5 per cent solution of 9, 10-dimethyl 1, 2-benzanthracene in benzene.

If enough Vitamin B is given the onset of warts is delayed, the area covered by lesions is smaller and the percentage producing definite tumours is lower than in the controls.

I am most grateful to Mr. F. A. Robinson, M.Sc., of Messers Allen & Hanbury for supplying both the yeast extract and the synthetic Vitamin B mixture. Without his help it would not have been possible for these experiments to have been carried out on a comparative basis.

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PHARMACOLOGICAL STUDIES ON THE EMBRYONAL STAGE ON THE VITAMIN C METABOLISM OF THE DEVELOPING HEN'S EGGS*

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INTRODUCTION

As part of the pharmacological studies of embryonal stage, Wada (1) of our Department investigated the influences of saccharin on the chick embryo, and confirmed that the "fertile egg injection method" is extremely advantageous to survey the activity and the fate of various substances in the living individuals. Employing this method, Wada conducted experiments concerning the toxicity of saccharin, its influences on the development of chick embryo, its diuretic activity, pathologic histologic investigation on various organs, and relation to the fate of saccharin and proteolytic enzymes of the liver, thereby he clarified the pattern of the excretion of saccharin. Further, Mori (2) performed a detailed investigation in the influences of *parotin* (a salivary gland hormone) on the development of chick embryo in general and on the development of bone of chick embryo, thereby he obtained an extremely interesting results.

When the author investigated the influences of various chemicals on the vitamin metabolism in the embryonal stage, this method was employed as it was done by Wada and Mori. As the first step, attempts were made to investigate the influences of various vitamins on the vitamin C metabolism in the embryonal stage. In the present experiment, the rise and fall of the vitamin C content in the course of incubation of fertile eggs without treatment was traced on the chick embryo as well as on various other portions of the eggs.

MATERIALS AND METHODS

Fertile eggs were obtained from a nearby poultry farm. They were laid by white leghorns. They were as fresh as 1—2 days old. Eggs of a roughly uniformed size (approximately 50 g in weight) were selected and incubated in an electrically regulated incubator at 38°C (103° F) and at the humidity of 64—65%. Utmost care was taken of the ventilation of the incubator. Eggs

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were taken out for experiments at various stages of development.

The primordium of the chick embryo generally appears approximately on the 3rd day of incubation. However, in the earlier stage, larger number of embryo is required for the estimation. Therefore, eggs were used for the experiment beginning from the 5th day of incubation when only small number of embryo is sufficient for the estimation.

During the developing stage of the chick embryo, the amniotic and allantoic fluids are clearly separable, and it is not too difficult task to separate without bymixture of other substances. Even so, procedures were conducted with great care. From the 6th day of incubation, the separation of allantoic fluid becomes easier, and it is thin fluid in the beginning, while it becomes slightly cloudy from approximately the 15th—17th day of incubation. Similarly to the allantoic fluid, the amniotic fluid was collected and employed for the experiments from the 6th day of incubation. Generally, the amniotic fluid is thin, but it becomes viscid in the later stage of the development.

First of all, the shell is broken in the middle of the egg, then the allantoic membrane which is holding the allantoic fluid is cut open to collect carefully the allantoic fluid without bymixture of other substance for experiment. The amniotic membrane is then cut open to collect amniotic fluid. Separately, part of the egg yolk is collected for the experiment. The eye balls of the embryo are next removed at the optic nerve bundle and employed for experiment separated from the embryo.

The former three (allantoic fluid, amniotic fluid and egg yolk) are quantitatively diluted to contain as high concentration as possible of vitamin C with metaphosphoric acid solution and distilled water. In the latter two cases (embryo and eye balls), the tissues are ground thoroughly and rapidly in a mortar with purified marine sand before they are diluted.

The estimation of vitamin C was conducted according to the indophenol method (3).

As to the calculation of day of incubation, the 1st day meant that the egg was placed in the incubator 24 hours or less ago. Thus, every 24 hours was counted 2nd, 3rd, and 4th day and so on.

The embryo on the 21st day means that it is 3 hours after a small hole was made by the embryo on the egg shell after the end of the 20th day of incubation. However, in this case, only the body weight was measured but the estimation of vitamin C was not conducted. Therefore, as soon as the time prescribed was attained, the egg was taken out from the incubator for the experiment.

Until the 5th to 10th day of incubation, materials from 3—5—10 eggs have to be pooled for the calculation of the estimated values of one group, because until such time, the weight of embryo and eye balls are too light and the

allantoic fluid and amniotic fluid are too scanty. In every experiment, the average value of 5-6 groups was calculated. After the 11th day of incubation, one group consisted of 1-3 eggs and the average value of 5 groups was calculated. However, for the eggs on the 5th day of incubation, the estimation was conducted only on the embryonal body. For the embryonal body and eye balls, the estimations were conducted until the 20th day, and on allantoic fluid, amniotic fluid and egg yolk until the 18th day of incubation.

RESULTS OF EXPERIMENTS

A. On the Embryonal Body Weight and Other Changes during the Incubation (See Table 1)

In the development of chick embryo, geometrically progressive increase in the body weight is generally seen in the last half of the incubation. For instance, the body weight on the 5th day is 0.4 g, whereas it is 2.8 g on the 10th day, showing an increase by approximately 7 times. The increase in the body weight after the 10th day is very sharp compared with that in the first half of the incubation. On the 18th day, the body weight is approximately 20 g, while on the 21st day, it is approximately 30 g, demonstrating an increase by approximately 10 g within the last three days.

The weight of the eye balls also increase in parallel with the increase in the weight of the embryonal body. In the first half of the incubation, it occupies 1/6 to 1/5 of the embryonal body, but it increases gradually during the course of development.

As to the changes in the general appearance, beginning from the 6th day, the cervical region becomes slender and the extremities begins to take forms. Also, the portion connecting the yolk sac and the embryo becomes thinner. In the embryo on the 8th day, the lower extremities looks markedly longer,

TABLE 1

Day of incubation	Egg count	Average weight of embryo (g)	Average weight of eye balls (g)	Average amount of allantoic fluid (g)
5	38	0.4		
6	35	0.7	0.15	1.3
7	25	1.0	0.20	1.8
8	27	1.5	0.30	2.5
9	24	2.4	0.40	3.0
10	20	2.8	0.45	3.5
11	12	3.6	0.50	3.5
12	10	4.9	0.55	3.4
13	12	7.4	0.60	5.2
14	8	9.0	0.65	6.0
15	9	13.4	0.70	8.1

16	8	14.8	0.75	4.5
17	10	17.2	0.80	4.0
18	8	19.4	0.80	3.8
19	8	22.5	0.80	
20	6	24.0	0.80	

the upper and lower bills are well developed, and the so-called egg-tooth is also found developing. On the 11th day, the growth and differentiation of the four extremities becomes further evident showing that the upper extremities are taking the form of the wings and the lower extremities are taking the form of the legs. Roughly on the 12th day of incubation, feathers begin to grow on the skin.

Relation to the allantoic and amniotic fluid, the pattern of the changes were described in the above. The egg yolk is viscid in the earlier stage, but the viscosity decreases gradually in the later stage, and further, viscid portion and less viscid portion are found intermingled. The volume of egg yolk also decreases gradually, and almost no egg yolk is found in the terminal stage of the incubation.

B. On the Change in the Vitamin C Content during the Incubation

It has already been reported by numerous earlier workers that the egg yolk and egg albumen contains no vitamin C or very little. G. H. Satterfield *et al.* (4) (1947) recognized that, even when a hen was given a total of 2150 mg of *l*-ascorbic acid in 21 weeks by 2—4 subcutaneous injections per week, the eggs laid by the hen do not contain any amount of vitamin C.

1. On the vitamin C content of chick embryo during the incubation (See Table 2)

The total vitamin C content of the chick embryo increases beginning from the 5th day (body weight 0.4 g, vitamin C 15.19 mg%) up to the 12th day of incubation (body weight 4.9 g, vitamin C 29.52 mg%), and gradually decreases later to 15—16 mg%. On the 20th day of incubation, the body weight is 24.0 g containing 16.43 mg% of vitamin C.

The correlationship between the reduced vitamin C content and oxidized vitamin C content in the chick embryo demonstrates that the former is always larger than the latter. Similar to the total vitamin C content, the reduced vitamin C content increases up to the 12th day of incubation (25.41 mg%) and gradually decreases later. On the 20th day of incubation, it is 13.85 mg%.

The oxidized vitamin C content increases beginning from the 6th day of incubation (3.60 mg%) up to the 9th day (6.63 mg%), and then reaches the bottom on the 11th day (1.76 mg%). It gradually increases again up to the 15th day (9.93 mg%) and then decreases again. On the 20th day of incubation, it is 2.58 mg%.

II. On the vitamin C content of eye balls during the incubation (See Table 2)

As stated in the above, it is characteristic that the weight of the eye balls is relatively heavy in the earlier stage. The total vitamin C content gradually increases during the incubation up to the 9th day of incubation (15.81 mg%) and then gradually decreases. Thereafter, it increases up to the 12th day (16.81 mg%) again, and then decreases to the bottom on the 17th day (10.40 mg%). However, a slight increase is seen thereafter. In the correlationship between the reduced vitamin C content and the oxidized vitamin C content, an equal value is seen in terms of mg% on or about the 8th day of incubation. But, up to approximately the 12th day thereafter, the reduced vitamin C content is larger than that of the oxidized vitamin C content. However, the oxidized vitamin C content becomes larger than that of the reduced vitamin C content at later date. Beginning from the 10th day (10.91 mg%), the reduced vitamin C content decreases to 3.5—4.5 mg%. Once on the 10th day, the oxidized vitamin C content decreases to 2.6—3.1 mg%, and thereafter, it increases gradually.

III. On the vitamin C content of allantoic fluid during the incubation (See Table 3)

The total vitamin C content of the allantoic fluid in terms of mg% increases as the incubation progresses. It reaches the peak on the 11th or 12th day of incubation (7.21—6.81 mg%), then after a transient decrease, it increases again. In the correlationship between the reduced vitamin C content and the oxidized vitamin C content, beginning from the 9th day of incubation, the oxidized vitamin C content becomes larger in the last half of the incubation.

During the whole course of incubation, the reduced vitamin C content demonstrates no marked rise and fall. It maintains the level of 1.5—2.5 mg%.

The oxidized vitamin C content changes roughly in the same manner as that of the total vitamin C content showing an increase in terms of mg%.

IV. On the vitamin C content of amniotic fluid during the incubation (See Table 3)

During the whole course of incubation, the total vitamin C content maintains the level of 2.0—3.0 mg%. Further, in the correlationship between the reduced vitamin C content and the oxidized vitamin C content, no remarkable difference is noted between the two. A slight increase is demonstrated in the reduced vitamin C content on about the 9th—10th day of incubation. Almost no fluctuation is noted in the oxidized vitamin C content during the whole course of incubation.

V. On the vitamin C content of egg yolk during the incubation (See Table 3)

The total vitamin C content increases gradually since the beginning of the incubation up to the 7th day (8.33 mg%) indicating that the egg yolk contains relatively large quantity of vitamin C. However, it gradually decreases in the last half of the incubation. Moreover most part of the total content is the oxidized vitamin C.

The reduced vitamin C content demonstrates no marked fluctuation during the whole course of incubation keeping the level of 0.5 — 0.8 mg%.

C. On the Influence of Blood Component Containing Tissues on the Estimated Values of Reduced Vitamin C Content

Generally speaking, in the cases of the tissues containing a large amount of blood component, the ascorbic acid is transformed to the oxidized vitamin C when the material is deproteinized with metaphosphoric acid, thus the value given as the reduced vitamin C content tends to be smaller than the actual value. Therefore, such influence was investigated on the materials from the embryo. For the purpose, one part of the material was deproteinized after the aeration with CO gas, and another part was deproteinized without such aeration. However, there were no difference in the values thus obtained. Consequently, in view of the above experiment, it is considered that influence of the blood component on the values of reduced vitamin C content obtained in the cases of the embryo, eye balls etc. was almost negligible.

TABLE 2. On the vitamin C content of chick embryo and eye balls during the incubation (mg%)

Portion Vitamin Day of incubation	Chick embryo			Eye balls		
	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C
5	15.19	9.73	5.46			
6	15.08	11.48	3.60	8.02	3.51	4.51
7	16.02	12.01	4.01	12.31	5.71	6.60
8	16.74	12.64	4.10	13.52	7.11	6.41
9	19.16	12.53	6.63	15.81	9.52	6.29
10	23.12	18.22	4.90	13.53	10.91	2.62
11	23.72	21.93	1.76	12.21	9.04	3.17
12	29.52	25.41	4.11	16.81	8.80	8.01
13	21.11	16.06	5.05	12.30	5.40	6.90
14	21.92	16.20	5.72	15.81	5.93	9.88
15	22.15	12.22	9.93	15.62	5.83	9.79
16	21.31	13.12	8.19	14.01	5.31	8.70
17	18.35	12.72	5.63	10.40	4.20	6.20
18	15.92	9.30	6.62	15.12	3.30	12.12
19	16.18	10.82	5.33	15.84	4.65	11.19
20	16.43	13.85	2.58	16.12	4.18	11.91

TABLE 3. On the vitamin C content of allantoic fluid, amniotic fluid and egg yolk during the incubation (mg%)

Portion Vitamin Day of incubation	Allantoic fluid			Amniotic fluid			Egg yolk		
	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C
6	2.68	0.94	1.70	3.41	2.34	1.07	5.21	0.28	4.93
7	3.84	1.95	1.89	2.32	1.31	1.01	8.33	0.34	7.99
8	3.23	1.84	1.44	2.38	1.43	0.95	7.22	0.46	6.76
9	3.15	1.68	1.47	3.19	2.71	0.48	5.73	0.56	5.17
10	4.75	1.75	3.00	3.01	2.52	0.49	5.87	0.52	5.35
11	6.52	2.05	4.47	1.92	1.30	0.62	4.10	0.64	3.46
12	7.21	2.32	4.89	2.11	1.31	0.80	4.70	0.60	4.10
13	6.84	2.58	4.31	1.65	0.78	0.87	6.25	0.63	5.62
14	5.83	1.71	4.12	2.39	1.42	0.88	5.83	0.64	5.19
15	5.21	2.20	3.01	1.92	0.88	1.04	5.63	0.84	4.79
16	6.90	1.07	5.83	1.50	0.82	0.68	4.95	0.76	4.19
17	5.90	0.57	5.33	1.18	0.55	0.63	3.78	0.65	3.13
18	5.21	0.53	4.71	1.43	0.51	0.92	4.71	0.59	4.12

CONCLUSIONS

1. The appearance of vitamin C in the chick embryo seems to be initiated simultaneously with the initiation of the incubation. The vitamin C content gradually increases. Moreover, in the case of embryo, the reduced vitamin C content is always larger than the other.

2. The vitamin C content in the eye balls fluctuates during the course of incubation. However, in the last half of the incubation, the oxidized vitamin C content is larger than the other.

3. In the case of allantoic fluid, the vitamin C content is inclined to a gradual increase. As soon as the vitamin C content began to increase, the oxidized vitamin C content becomes larger than the other.

4. In the case of amniotic fluid, the vitamin C content does not show any marked fluctuation during the whole course of incubation.

5. The total vitamin C content in the egg yolk is relatively larger in the first half of the incubation, but it gradually decreases in the last half of the incubation. Furthermore, most part of the vitamin C is oxidized type.

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PHARMACOLOGICAL STUDIES ON THE EMBRYONAL STAGE ON THE INFLUENCE OF VITAMINS C, B₁ AND S-B₁ ON THE VITAMIN C METABOLISM IN THE DEVELOPING HEN'S EGGS*

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INTRODUCTION

Surendra Nath Ray (1) reported that, when hen's eggs are artificially incubated, the vitamin C content increases as the embryo develops. Satterfield, *et al.* (2) reported that vitamin C is not contained in the hen's eggs laid by a hen injected with L-ascorbic acid. Okano (3) recognized that vitamin C synthesis in the hen's eggs becomes remarkable since approximately the 15th day of incubation, and that this tendency is specifically evident in the case when sugars were injected. On the other hand, Sasamoto (4) confirmed that egg albumen and egg yolk before the incubation lack vitamin C when he investigated the correlation between vitamin C of egg albumen, egg yolk and embryo during the incubation and dihydro-gulonic acid. In the previous paper (5), the author recognized that the appearance of vitamin C takes place simultaneously with the initiation of incubation when he investigated the vitamin C metabolism of untreated hen's eggs. Further, he traced the pattern of the distribution of vitamin C in the chick embryo, eye balls, allantoic fluid, amniotic fluid and in the egg yolk.

In the present paper, the author attempts to trace the changes in the distribution of vitamin C by investigation the influence of vitamin C, B₁ and S-B₁ on the vitamin C metabolism in the developing hen's eggs, in addition to the investigation of the toxicity of these vitamins to the embryo and the influence of these vitamins on the development of the embryo in general.

MATERIALS AND METHODS

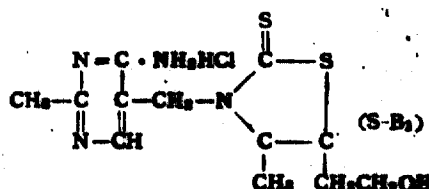
The materials and methods for the present work were all in accordance with those of the work described in the previous paper (5), unless otherwise described in the results of the experiments.

*Director: Prof. Sô. Tameo

Vitamins were administered according to the "egg injection method." The surface of a fertile egg, especially of the apex of the egg was cleansed with alcohol, and a small hole was bored with a gimlet, and through this hole, solution of vitamin was introduced with an injection syringe. The small hole was then closed with a small strip of paper soaked in paraffin.

One injection of 0.1 cc was given on the 5th day of incubation.

Vitamin preparations employed for the experiments were Vitamin C Injection (Tanabe), Vitamin B₁ Hydrochloride (Takeda) and Vitamin S-B₁ Hydrochloride (Takeda).



RESULTS

A. Experiments with Vitamin C Application (See Table I)

On the 5th day incubation, 5 mg (in 0.1 cc) of vitamin C was injected into fertile hen's eggs, and the vitamin C contents in terms of mg% of the embryo, eye balls, allantoic fluid, amniotic fluid and egg yolk were estimated on the 6th, 7th, 8th, 9th and the 10th day of incubation.

TABLE I. C

Day of incubation	Egg count	Average weight of embryo (g)	Average weight of eye balls (g)	Average weight of allantoic fluid (g)
6	28	0.5	0.15	1.4
7	25	0.9	0.20	1.6
8	18	1.3	0.30	2.6
9	27	2.2	0.40	2.9
10	18	2.5	0.65	3.3

I. Cases of chick embryo (See Table 2)

The total vitamin C content showed a sharp rise after the injection. On the 5th day before the injection, it was 15.19 mg%, while it was 18.43 mg% after 24 hours, and on the 7th day, it decreased slightly (18.00 mg%). However, during the 8th, 9th and the 10th day, it was 20.10 mg%, 24.38 mg% and 30.32 mg% respectively, showing a relatively rapid rise.

Although the reduced vitamin C content showed not too remarkable increase, it demonstrated a tendency of increase in terms of mg%. Contrary to this, a rapid increase in the oxidized vitamin C content was noted. It was 3.05 mg%

after 48 hours, 5.89 mg% after 72 hours, 8.04 mg% after 4 days, and 14.20 mg% after 5 days. Moreover, the reduced vitamin C content was always larger than the oxidized vitamin C content.

II. Cases of yolk balls (See Table 2)

After the injection, an evident increase in the total vitamin C content was observed. It was 18.64 mg% on the 7th day, while it was only 12.31 mg% in the control cases on the same day. In the injected cases, it increased rapidly thereafter, and it was 24.97 mg% on the 10th day, while it was 13.53 mg% in the control cases on the same day. On the 8th and 10th day, the oxidized vitamin C content was larger than the other.

III. Cases of allantoic fluid (See Table 3)

The total vitamin C content after 24 hours was 4.35 mg% showing a slight increase. The content decreased thereafter, and it became 2.12 mg% on the 10th day. The oxidized vitamin C content became larger than the other beginning from 48 hours after the injection.

IV. Cases of amniotic fluid (See Table 3)

The total vitamin C content after 24 hours was 5.05 mg%, and it maintained the level of 3—4 mg% thereafter. The oxidized vitamin C content was larger than the reduced vitamin C content.

V. Cases of egg yolk (See Table 3)

The total vitamin C content after 24 hours was 6.15 mg%. It was 6.42 mg% after 48 hours, 8.04 mg% after 3 days, 8.89 mg% after 4 days and was 6.95 mg% after 5 days. The reduced vitamin C content was smaller, and most part was occupied by the oxidized vitamin C content.

B. Experiments with Vitamin B₁ Application (See Table 4)

Similarly to the application with vitamin C, 100γ (in 0.1 cc) of vitamin B₁ hydrochloride was injected into fertile hen's eggs on the 5th day of incubation. These eggs were used daily for the estimation of vitamin C content of the chick embryo, eye balls, allantoic fluid, amniotic fluid and egg yolk beginning from

TABLE 2. Experiments with vitamin C (5 mg) application (mg%) (Chick embryo and eye balls)

Day of Incubation	Chick embryo			Eye balls		
	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C
6	18.63	14.63	3.97	6.23	5.95	0.96
7	18.00	14.95	3.05	15.44	9.17	6.47
8	21.10	14.21	5.89	23.87	9.63	11.17
9	21.33	16.34	8.04	21.08	10.81	9.27
10	20.32	16.02	14.33	21.97	10.83	14.03

the 6th day (24 hours after the injection) up to the 10th day of incubation. Thereafter, the same estimation was conducted on the 12th, 15th, 18th and on the 20th day of incubation. However, estimations on the 20th day of incubation were conducted only on the chick embryo and eye balls.

TABLE 3. Experiments with vitamin C (5 mg) application (mg%)
(Allantoic fluid, amniotic fluid and egg yolk)

Portion Vitamin Day of incubation	Allantoic fluid			Amniotic fluid			Egg yolk		
	Total C	Reduced C	Oxidised C	Total C	Reduced C	Oxidised C	Total C	Reduced C	Oxidised C
6	4.35	2.47	1.88	5.05	1.89	3.16	6.15	0.92	5.23
7	2.78	1.13	1.65	2.03	0.82	1.21	6.42	0.29	6.13
8	2.98	0.65	2.32	2.91	0.95	1.96	8.04	0.33	7.71
9	2.27	0.59	1.68	4.35	0.34	4.01	8.60	0.43	8.17
10	2.12	0.89	1.23	2.61	0.59	2.02	6.95	0.60	6.35

TABLE 4. B₁₂

Day of incubation	Egg count	Average weight of embryo (g)	Average weight of eye balls (g)	Average amount of allantoic fluid (g)
6	21	0.4	0.10	1.3
7	23	0.7	0.18	1.5
8	21	1.4	0.28	2.2
9	18	2.0	0.35	1.8
10	16	2.5	0.50	2.0
12	12	5.7	0.62	2.7
15	8	11.7	0.75	6.8
18	10	19.2	0.78	4.9
20	6	21.5	0.80	—

I. Cases of chick embryo (See Table 5)

The total vitamin C content gradually increased after the injection. It was 15.70 mg% after 24 hours, 21.28 mg% after 3 days, 23.80 mg% after 5 days (the 10th day of incubation) and it was 28.87 mg% on the 12th day of incubation. It decreased thereafter, and it was 15.76 mg% on the 20th day of incubation. The reduced vitamin C content was 10.49 mg% on the 6th day of incubation. It demonstrated a tendency of gradual increase thereafter, and reached 20.15 mg% on the 12th day of incubation. It was 14.27 mg% on the 15th day, 13.95 mg% on the 18th day and 11.31 mg% on the 20th day of incubation showing a decrease in terms of mg%. Similar rise and fall showing the similar tendency was seen in the oxidised vitamin C content. Through the whole course of incubation, the reduced vitamin C content was larger than oxidised vitamin C content.

II. Cases of eye balls (See Table 5)

The total vitamin C content 24 hours after the injection (on the 6th day of incubation) was 14.45 mg%, and it was 19.12 mg% after 48 hours. It increased thereafter to reach 21.80 mg% on the 10th day of incubation. A transient decrease was seen before it increased again from the 15th day to reach 22.56 mg% on the 18th day of incubation. In the reduced vitamin C content, it increased in terms of mg % after the injection up to the 10th day showing the value of 12.27 mg%. Thereafter, it decreased to show the value of 5.08 mg%. The oxidized vitamin C content was 8.48 mg% on the 6th day, and it was 11.77 mg% on the 9th day. A gradual decrease thereafter was seen before it began

TABLE 5. Experiments with vitamin B₁ (100 γ) application (mg%)
(Chick embryo and eye balls)

Portion Day of incubation	Chick embryo			Eye balls		
	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C
6	15.70	10.40	5.21	14.45	5.97	8.48
7	21.11	13.72	7.39	19.12	9.03	10.09
8	21.28	15.11	6.17	20.81	10.11	10.73
9	22.69	17.55	5.14	21.65	9.69	11.77
10	23.60	18.22	6.38	21.80	12.27	9.53
12	20.67	20.16	8.71	19.50	12.15	7.45
15	23.04	14.27	8.77	15.98	9.83	6.15
18	20.21	13.95	6.26	22.56	5.63	10.93
20	15.76	11.31	4.45	20.69	5.08	15.61

to increase again from the 15th day to reach 15.61 mg% in terms of mg% on the 20th day of incubation.

III. Cases of allantoic fluid (See Table 6)

The value was 7.40 mg% 24 hours after the injection, 5.74 mg% after 48 hours and was 5.14 mg% after 4 days. It was 6—7 mg% on the 12—15th day of incubation. The reduced vitamin C content was maintained at the level of 2—4 mg% during the whole course of incubation without marked rise or fall. The oxidized vitamin C content maintained the level of roughly 3 mg% up to approximately the 10th day of incubation, but it decreased thereafter.

IV. Cases of amniotic fluid (See Table 6)

The total vitamin C content in terms of mg% showed a slight increase after the injection. It reached 8.42 mg% on the 8th day of incubation, and showed a decrease thereafter. The reduced vitamin C content showed a decrease as the incubation went on. In the oxidized vitamin C content, almost no rise or fall was noted during the whole course of incubation. The oxidized vitamin C content was always found to be larger.

V. Cases of egg yolk (See Table 6)

The total vitamin C content was found gradually increasing after the injection up to the 10th day of incubation. It was 5.24 mg% after 24 hours and was 7.70 mg% after 5 days (on the 10th day of incubation). The reduced vitamin C content maintained the level at 1.0—2.0 mg% during the whole course of incu-

TABLE 6. Experiments with vitamin B₁ (100 r) application (mg%)
(Allantoic fluid, amniotic fluid and egg yolk)

Portion Vitamin Day of incubation	Allantoic fluid			Amniotic fluid			Egg yolk		
	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C
6	7.40	3.59	3.81	4.78	3.05	1.73	5.24	1.01	4.23
7	5.74	3.44	2.30	5.59	3.45	2.13	6.15	1.05	5.07
8	5.77	2.69	3.17	6.42	3.11	3.31	5.94	1.14	4.80
9	5.14	1.05	3.49	4.12	1.81	2.31	6.80	1.16	5.64
10	4.63	1.00	3.63	3.89	2.34	1.55	7.70	1.89	5.72
12	7.78	2.78	5.00	2.94	1.48	1.46	5.41	0.88	4.49
15	6.24	4.85	1.39	3.00	2.10	0.90	5.52	0.88	4.66
18	4.48	2.62	1.84	1.85	1.14	0.71	4.84	0.85	4.29

bation almost without rise or fall. Similar tendency was noted in the oxidized vitamin C content, which maintained the level of 4—5 mg%. The oxidized vitamin C content was always found to be larger than the other.

C. Experiments with Vitamin S-B₁ Application (See Table 7)

Fertile eggs were injected with 100 r (in 0.1 cc) of vitamin S-B₁ hydrochloride in a same manner as in the case of vitamin B₁ application on the 5th day of incubation. They were employed for serial experiments.

I. Cases of chick embryo (See Table 8)

The total vitamin C content was found to be gradually increasing after the injection. It was at the level approximately 20 mg% 24—48 hours after the injection, and was approximately 25—26 mg% on the 10th—12th day of incubation. Thereafter, it was found slightly decreased, showing the value of 22.45 mg% on the 20th day of incubation. The reduced vitamin C content was found gradually increased on the 10—12th day of incubation showing the value of approximately 21 mg%. It was 17.95 mg% on the 12th day, 13.25 mg% on the 15th day and was 16.89 mg% on the 20th day of incubation. The oxidized vitamin C content was found to be maintaining the level at approximately 5—6 mg% until 5 days from the injection. It was 7.92 mg% on the 15th day, 10.24 mg% on the 18th day and was 6.85 mg% on the 20th day of incubation. Relatively, the reduced vitamin C content was found always to be larger than the oxidized vitamin C content.

TABLE 7. S-B₁

Day of incubation	Egg count	Average weight of embryo (g)	Average weight of eye balls (g)	Average amount of allantoic fluid (g)
6	32	0.4	0.15	1.8
7	28	0.7	0.20	2.2
8	25	1.4	0.30	2.5
9	20	2.4	0.35	2.6
10	16	3.0	0.45	3.4
12	10	4.7	0.60	5.3
15	8	12.0	0.65	7.4
18	12	20.8	0.75	4.5
20	6	23.1	0.75	—

II. Cases of eye balls (See Table 8)

The total vitamin C content was 22.04 mg% 5 days after the injection. Thereafter, it was found to be decreasing in terms of mg% showing the value of 18.59 mg% on the 20th day of incubation. The reduced vitamin C content increased up to the 10th day of incubation. It was 8.02 mg% 24 hours after the injection and was 12.22 mg% 5 days after the injection. It decreased thereafter and was 5.55 mg% on the 20th day of incubation. The oxidized vitamin C content was 7.76 mg% 24 hours after the injection, and was approximately 9—10 mg% and on the 10—15th day of incubation. On the 18th day of incubation, a slight further increase was seen. Relatively, the reduced vitamin C content was larger than the oxidized vitamin C content during the period of 9th and the 15th day of incubation, but the latter was larger thereafter.

TABLE 8. Experiments with vitamin S B₁ (100 r) application (mg%)
(Chick embryo and eye balls)

Portion Vitamin Day of incubation	Chick embryo			Eye balls		
	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C
6	20.68	13.56	6.52	15.78	8.02	7.76
7	19.91	14.73	5.18	16.40	9.32	7.08
8	23.05	17.66	5.39	19.80	9.84	9.96
9	24.24	17.54	6.70	20.05	10.69	9.36
10	26.85	21.26	5.59	22.04	12.22	9.82
12	25.29	21.15	4.14	21.08	11.21	9.84
15	24.97	17.05	7.92	18.43	9.30	9.13
18	23.57	13.23	10.31	19.49	5.25	14.24
20	23.44	16.59	6.85	15.59	5.55	10.04

III. Cases of allantoic fluid (See Table 9)

The total vitamin C content showed no marked fluctuation during the whole

course of incubation maintaining the level at approximately 5—6 mg%. Also the reduced vitamin C content and the oxidized vitamin C content maintained the level at approximately 1—3 mg% and 3—4 mg% respectively.

IV. Cases of amniotic fluid (See Table 9)

The total vitamin C content was 5.28 mg% 24 hours after the injection, and was 3.99 mg% after 48 hours. It was found gradually decreased thereafter showing the level at approximately 2—3 mg% during the period of the 10th—20th day of incubation.

TABLE 9. Experiments with vitamin S-B₁ (100 r) application (mg%)
(Allantoic fluid, amniotic fluid and egg yolk)

Portion Day of incubation	Allantoic fluid			Amniotic fluid			Egg yolk		
	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C	Total C	Reduced C	Oxidized C
6	5.60	1.50	4.10	5.28	2.97	2.31	4.18	1.24	2.94
7	4.90	1.16	3.74	3.99	2.42	1.57	5.82	1.36	4.46
8	4.88	1.68	3.20	3.23	2.05	1.18	5.70	1.30	4.40
9	4.58	1.37	3.21	4.56	3.86	0.70	7.23	0.85	6.38
10	6.25	1.93	4.32	3.02	1.20	1.82	6.27	0.92	5.35
12	5.66	1.63	4.03	3.04	1.20	1.84	3.01	0.89	2.12
15	6.45	3.32	3.13	2.79	1.69	1.10	7.06	0.53	6.53
18	5.36	2.28	3.08	2.09	1.40	0.69	5.12	0.68	4.34

V. Cases of egg yolk (See Table 9)

The total vitamin C content was 4.18 mg% 24 hours after the injection, and it was 7.23 mg% 4 days after the injection. It maintained thereafter the level ranging from 3 to 7 mg%. Relatively, the oxidized vitamin C content was always found larger than the reduced vitamin C content.

SUMMARY

As stated above, the body weight of chick embryo shows a rapid increase in the last half of the incubation, and further, even when the embryo was administered with vitamins C, B₁ or S-B₁, no particular discrepancies were seen compared with the control cases (untreated). In other words, in the application with 5 mg of vitamin C, 100 r of vitamin B₁ or S-B₁, the chick embryo developed very well and no incidences of malformation were recognized.

The average weight of the eye balls was found relatively large compared with the body weight of the chick embryo in the earlier stage of the incubation occupying 1/6—1/5 of the body weight. Also in this case, it was learnt that the application with either of the above three vitamins does not affect the development. The quantity of the allantoic fluid is relatively small in the

earlier stage of the incubation, but it is found increased on approximately the 12th—15th day of incubation. Either of the above three vitamins seemed not particularly to affect the quantity of the allantoic fluid. In view of the experiment by Wada (6) of our Department, in which he recognized the increase in the quantity of the allantoic fluid of the incubated fertile hen's eggs injected with saccharin, the above facts is considered to be suggesting that vitamin C, B₁, etc. do not affect the movement of watery component in the embryonal stage.

Following the above, investigation was conducted in the influence of the vitamin C application on the vitamin C metabolism in the embryonal stage.

In the case of chick embryo, the total vitamin C content in terms of mg% or in total amount was found higher compared with the case of untreated control. Though the oxidized vitamin C content on the 8th—10th day of incubation was higher in the cases of vitamin C application compared with untreated control, it did always not reach the level of the reduced vitamin C content. Also in the case of eye balls, the total vitamin C content on the 8th—10th days of incubation in the vitamin C application group exceeded that of the untreated group. However, the increase is solely due to the increase in the oxidized vitamin C content. The total vitamin C content of the allantoic fluid was relatively large 24 hours after the injection, but it was found decreased in the total amount on the 10th day of incubation on the contrary. As to the amniotic fluid and egg yolk, the vitamin C content in terms of mg% did not show any marked difference compared with the untreated group, but a slight increase in the vitamin C content was observed in the applied group 24 hours after the treatment.

The total vitamin C content of the embryo applied with vitamin B₁ or with vitamin S-B₁ showed no marked difference with that of the control group up to the 12th—15th day of incubation, but a slight increase in the case of S-B₁ applied group thereafter. The vitamin B₁ introduced group showed roughly the same tendency as the untreated control group. The total vitamin C content of the eye ball demonstrated a tendency to an increase in terms of total amount by vitamin B₁ or vitamin S-B₁ application. Relatively, the reduced vitamin C content was found slightly larger up to approximately the 12th day of incubation, but the oxidized vitamin C content exceeded the other thereafter. The transfer of vitamin C into the allantoic fluid was found increased in terms of total amount as the quantity of the allantoic fluid increased, but no marked influence due to the vitamin B₁ or vitamin S-B₁ was recognized. Relatively, the oxidized vitamin C content was found slightly larger than the other approximately on the 10th—12th day of incubation, but these two were found to be excreted in the same degree thereafter.

In short, it is considered that the application with vitamin B₁ or with

vitamin S-B₁ in such an amount as 100 γ does not affect the vitamin C metabolism of the chick embryo demonstrating only a general stimulating effect as a foreign body. It was a matter of interest that the vitamin C applicated was transferred to the chick embryo, thus the vitamin C content of the eye balls was found increased clearly indicating that the vitamin C introduced was transferred to the eye balls. In respect to the above, in view of the fact that the vitamin C content of the chick embryo and the eye balls was found increased immediately after the vitamins were applicated, and that the vitamin C content of the allantoic fluid, which is considered to be the location for the excretion of excessive substance, was found increased in roughly the same amount as that of the untreated control group, the previous statement is considered to be supported.

CONCLUSIONS

1. The chick embryo develops very well without incidence of malformation under the application with vitamin C (5 mg), vitamin B₁ (100 γ) or with vitamin S-B₁ (100 γ).

2. Vitamin C, B₁ or S-B₁ does not give any active influence on the movement of watery component in the embryonal stage.

3. It is difficult to find any difference in the toxicity of vitamin B₁ and vitamin S-B₁ to the chick embryo.

4. It is presumed that part of the introduced vitamin C is transferred to the chick embryo, especially to the eye balls remarkably, and stored there.

5. The total vitamin C content of the chick embryo and eye balls in terms of mg% increased beginning from 1—2 days after the application. However, the value of the vitamin C content of the allantoic fluid maintains roughly the same value of that of the untreated control group without increase. (It was found even decreased on the 10th day of incubation.)

6. The vitamin C metabolism of the chick embryo seems not to be particularly influenced by the application with vitamin B₁ or vitamin S-B₁ in such an amount as 100 γ up to the 12th—15th day of incubation, but a slight increase in the total vitamin C content of S-B₁ applicated group thereafter. In the case of eye balls, the vitamin C content is found increased by the application with vitamin B₁ or with vitamin S-B₁.

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An Antithyroid Effect of Thiamine

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ABSTRACT. Rat thyroid slices incubated in the presence of radioiodine and thiamine ($10^{-3}M$) contained less organic radioiodine than control slices. There was no effect of thiamine, however, on the radioiodine content of slices in the presence of propylthiouracil. Thiamine in the diet of rats occasionally inhibited the formation of organic radioiodine compounds but, when augmented by injections of thiamine, inhibition

became a consistent finding. There was no goiter formation or increase in the ratio of radiomonoiodotyrosine to radiodiodotyrosine. It is concluded that thiamine has an antithyroid effect similar to propylthiouracil in inhibiting the formation of monoiodotyrosine although it may not inhibit the later steps in thyroid biosynthesis. (*Endocrinology* 82:895, 1968)

ALTHOUGH treatment with thiamine has had a beneficial effect in animals made thyrotoxic with thyroid (1) and in humans with hyperthyroidism (2), the effect, in these instances, has been said to be a result of the correction of a relative deficiency of the vitamin. It has recently been stated that "thiamine has no antithyroidal effect" (3).

This study presents evidence that thiamine has an antithyroid effect both *in vitro* and *in vivo* and that this effect is different in some respects from that of propylthiouracil.

Materials and Methods

In vitro experiments. Calf thyroid slices (140-160 mg) were added to 2 ml of Krebs-Ringer-Tris buffer (pH 7.4) containing 0.5 μ g of carrier potassium iodide and 2-20 μ Ci of radioiodide and incubated for 1 hr. The slices were removed, rinsed in fresh medium, counted and, in some instances, homogenized, incubated for 18 hr in 0.5 ml medium (pH 7.4) containing propylthiouracil ($10^{-3}M$), 1 drop of toluene and 10 mg pancreatin (N. F., Fisher), chromatographed in butanol-acetic acid-water (75:10:15) and radioautographed; appropriate sections of the paper were counted. The uptake of radioiodine by control slices was 6-17%. To experimental flasks was added thiamine hydrochloride ($10^{-3}M$ or $10^{-2}M$), or neopyrithiamine hydrobromide

($10^{-3}M$ or $10^{-2}M$) or oxythiamine hydrochloride ($10^{-3}M$ or $10^{-2}M$). In some experiments, sodium thiocyanate ($10^{-3}M$) was added for an additional $\frac{1}{2}$ hr of incubation to both control and experimental flasks. In other experiments, propylthiouracil ($10^{-3}M$) was present in both control and experimental flasks and thiamine hydrochloride ($10^{-3}M$ or $10^{-2}M$) in the experimental flasks. The addition of thiamine hydrochloride ($10^{-3}M$) to the proteolytic medium of pancreatin had no effect on the proteolysis.

In vivo experiments. Rats were fed diets (1.2 μ g I/g) containing 0.1-10 g/kg of thiamine hydrochloride for 4-15 days, given 5 μ Ci ^{131}I , and killed 2 or 4 hr later. Thyroids were removed, weighed, counted, homogenized, pancreatinized and chromatographed as above. The uptake of radioiodine by control thyroids was 5-10%. In some experiments, thiamine (20-60 mg) was given intraperitoneally in either single or multiple doses.

Results

The presence of thiamine hydrochloride ($10^{-3}M$ or $10^{-2}M$) in the medium in which calf thyroid slices were incubated reduced the amount of radiomonoiodotyrosine, radiodiodotyrosine and organic radioiodine formed by the slices (Table 1). When the higher concentration ($10^{-2}M$) was used, a similar effect was produced by both oxythiamine and pyrithiamine.

When the formation of organic iodine

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TABLE 1. Effect of thiamine, oxythiamine or pyrithiamine on the formation of organic radioiodine compounds by calf thyroid slices

Expt.	Substance	M ¹³¹ IT cpm/mg	D ¹³¹ IT cpm/mg	M ¹³¹ IT/D ¹³¹ IT	% Reduction org. ¹³¹ I
1	control (15) thiamine ⁻¹ (15)	7.5 2.0 (<0.001)	8.6 1.0 (<.025)	2.1 2.0	57 (<0.005)
2	control (20) thiamine ⁻¹ (20)	2.6 1.4 (<0.001)	1.1 0.7 (<0.001)	2.2 2.0	34 (<0.001)*
3	control (15) oxythiamine ⁻¹ (15)	7.5 2.3 (<0.005)	8.6 1.0 (<0.025)	2.1 2.3	62 (<0.01)
4	control (30) oxythiamine ⁻¹ (30)	5.5 5.2 (>0.5)	2.8 2.3 (>0.1)	2.0 2.3	15 (>0.05)
5	control (28) pyrithiamine ⁻¹ (28)	11.3 5.3 (<0.001)	4.7 4.0 (<0.05)	2.4 1.3	27 (<0.001)

% reduction org. ¹³¹I is the total amount of organic radioiodine (all the radioactivity on the slice other than that at the origin and the iodide spot) as a per cent of the control subtracted from 100%. The superscripts ⁻¹ and ⁻² mean 1×10^{-4} M and 1×10^{-5} M.

The figures in parentheses are the numbers of flasks compared or the p values determined by using test for paired differences.

* When the radioactivity at the origin is also included in the calculations as organic, this figure does not change.

compounds was inhibited by the presence of propylthiouracil (10^{-4} M), there was no effect of thiamine on the amount of radioiodide in the thyroid slice (652 cpm/mg vs. 658 cpm/mg; $p < 0.5$).

Occasionally the thyroids of rats fed thiamine contained less organic radioiodine than controls but the differences in grouped results of five experiments were not significant (Table 2). When thiamine was given in the diet and also injected daily, the uptake of radioiodine and the formation of organic radioiodine compounds were significantly reduced, although there was no change in the ratio of radiomonoiodotyrosine to radiodiiodotyrosine nor any increase in gland weight (Table 3).

Discussion

Both *in vitro* and *in vivo* thiamine had an

inhibitory effect on the formation of organic radioiodine compounds by the thyroid. It had no effect on the concentration of radioiodide in slices in which the formation of organic iodine compounds was blocked by propylthiouracil. Although these effects were similar to those of propylthiouracil and presumably other thiocarbamides, they differed in that thiamine reduced the formation of organic radioiodine compounds without increasing the ratio of radiomonoiodotyrosine to radiodiiodotyrosine. This finding suggests that unlike propylthiouracil (4), thiamine does not inhibit the conversion of radiomonoiodotyrosine to radiodiiodotyrosine. As an increase in the ratio of radiomonoiodotyrosine to radiodiiodotyrosine often occurs when the amount of iodine available for combination with tyrosine is reduced (5).

TABLE 2. Effect of dietary thiamine on the formation of organic radioiodine compounds by the thyroids of rats

Expt.	Substance	M ¹³¹ IT cpm/mg	D ¹³¹ IT cpm/mg	M ¹³¹ IT/D ¹³¹ IT	Gland wt mg/rat
1	control diet (4) thiamine 1 g/kg (4)	157 49 (<0.05)	270 92 (<0.05)	.6 .5	
2	control diet (20) thiamine 1 g/kg (20)	164 108 (>0.1)	197 120 (>0.05)	.8 .9	11 10

The figures in parentheses are the numbers of rats or the p values. Expt. 1 is a single experiment. Expt. 2 is a group of 5 experiments including Expt. 1.

TABLE 3. Effect of thiamine (in the diet supplemented by injection) on the formation of organic radiiodine and uptake of radiiodine by the thyroids of rats

Substance	M ¹²⁵ IT ⁶⁶ cpm/mg	D ¹²⁵ IT ⁶⁶ cpm/mg	M ¹²⁵ IT/D ¹²⁵ IT	Total ¹²⁵ I cpm/mg	% uptake* ¹²⁵ I	Gland wt mg/rat
control diet (8)	87	120	.7	8045	100	15
thiamine diet + ip (8)	56 (<0.05)	73 (<0.05)	.8	4662 (<0.025)	58	12
control diet (6)	293	424	.7	6919	100	13
thiamine diet + ip (6)	195 (<0.025)	255 (<0.025)	.8	4709 (<0.025)	68	12

The figures in parentheses are the numbers of rats or the p values. The thiamine was given as 1 g/kg and as 50 mg ip on each of the 5 days of the experiment (in the second experiment the ip dose was 25 mg twice a day).

* For ease of comparison the control uptake is arbitrarily set at 100%.

⁶⁶ M¹²⁵IT and D¹²⁵IT are about 30 and 40%, respectively, of the total ¹²⁵I on the strip.

† This represents the counts of the whole thyroids prior to hydrolysis and cannot be compared to the preceding columns in this table.

the absence of such a finding here may mean that thiamine compensates for such an effect by a stimulation further along the pathway of thyroxine biosynthesis. On the other hand, it now seems clear that all the radiiodotyrosine in the thyroid is not necessarily derived from radiomonoiodotyrosine (6) and the meaning of the ratio of these compounds is complex and unclear.

It is of interest that the large doses of thiamine used in these experiments did not cause enlargement of the thyroid. After the completion of this study, an article was found reporting goitrogenesis in the rat following the administration of microgram amounts of thiamine (7). It is possible that the large amounts used in the present study inhibited goiter formation which might have resulted had smaller doses been used.¹

The similar antithyroid effect of the analogues of thiamine (pyrithiamine, in which the thiazole ring of thiamine has been replaced by pyridine, and oxythiamine, in which the amine has been replaced by a hydroxy group on the pyrimidine

ring) raises a question as to whether it is the intact thiamine molecule and/or some derivative which exerts this effect. This cannot be answered. The major excretory products of thiamine metabolism in man are pyramin (a pyrimidine compound) and thiamine. *In vitro* mild oxidation converts thiamine to thiochrome. Other changes, in a compound which is similar in certain respects to known antithyroid compounds, may result in the formation of trace amounts of a compound which fits into an established antithyroid group (8).

Thiamine, then, represents another naturally occurring substance with an inhibitory effect on thyroid hormone biogenesis.

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¹ In a single experiment, thiamine in small amounts (20 µg) was injected twice daily for 17 days but no goiter resulted.

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In vivo and in vitro penetration of vitamins into human red blood cells^{1,2}

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Red blood cells have high concentrations of riboflavin, nicotinate, pantothenate, and reduced folates; vitamins A and E and oxidized folates are virtually undetectable when compared with plasma levels (1). After intravenous multivitamins, large increases of biotin, pantothenate, B₆, thiamin, and N⁵-methyltetrahydrofolate (N⁵-methyl-THF) were observed in red blood cells. No increases occurred in red blood cell ascorbate in oxidized or reduced folate, or in vitamins A and E. Presumably these vitamins quickly disappeared into tissues, with lowered availability for entry into the red blood cell, or in vivo the red blood cell membrane does not permit entry of some vitamins into the red cell. To test such assumptions we studied penetration of vitamins into red blood cells, in vitro as well as in vivo, to eliminate vitamin distribution into other tissues, and to avoid other factors operating in vivo that could affect entry of vitamins into red blood cells.

Methods

Studies were conducted with healthy, adult male volunteers chosen from laboratory personnel. Each had a normal hematocrit measurement, white blood cell count, peripheral smear, and reticulocyte count <1%; all had normal circulating vitamin titers (2).

In vivo

Six volunteers were used for this study. Blood was drawn into vacutainers (Becton, Dickinson & Co., Rutherford, New Jersey) containing 0.2 ml 25% sodium citrate, before, 3 hr after, and 24 hr after intravenous multivitamin administration, so as to monitor the influx and disappearance of vitamins into and from red cells. All vitamins were administered intravenously, except for thiamin propyldisulfide; 60 mg were given orally on a separate occasion since no intravenous preparation was available. Multivitamin preparations consisted of 2 ml of Borecca-C (Hoffmann-La Roche Laboratories, Nutley, New Jersey) plus 10 ml M.V.I. (USV Pharmaceutical Corp., New York, New York). This

amounted to 600 mg ascorbic acid; 60 mg thiamin HCl; 20 mg sodium riboflavin phosphate; 180 mg nicotinamide, 35 mg pyridoxol HCl; 45 mg D-pantothenol (equal to 32.2 mg Ca pantothenate); 0.2 mg biotin; 3 mg vitamin A; 1,000 USP units vitamin D; and 5 mg vitamin E. On separate occasions, other volunteers received either 60 mg thiamin pyrophosphate (cocarboxylase, TPP; Barrows Biochemical Products Corp., Inwood, New York), or 5 mg pteroylmonoglutamic acid (Folvite, folic acid, PGA; Lederle Laboratories, Pearl River, New York), or 10 mg *dl*-L-N⁵-formyltetrahydrofolic acid (Leucovorin, folinic acid, N⁵-formyl-THF; Lederle Laboratories), or 10 mg pteroyltriglutamate (Teroplerin; Lederle Laboratories), or 100 µg cyanocobalamin, or 100 µg hydroxocobalamin.

In vitro

Six volunteers were used for this study. Samples of fresh whole blood were each incubated for 3 and 24 hr at 37 C with each of the following: pyridoxol HCl 10 µg/ml; pyridoxal HCl 10 µg/ml; pyridoxamine 2HCl 10 µg/ml; pyridoxal-5-phosphate 10 µg/ml; nicotinic acid 30 µg/ml; nicotinamide 30 µg/ml; thiamin HCl 10 µg/ml; thiamin pyrophosphate 10 µg/ml; thiamin propyldisulfide 10 µg/ml; calcium pantothenate 10 µg/ml; sodium riboflavin phosphate 10 µg/ml; pteroylmonoglutamate 1 µg/ml; N⁵-formyl-THF 2 µg/ml; pteroyltriglutamate 2 µg/ml; N⁵-methyl-THF 1 µg/ml; cyanocobalamin 20 ng/ml; hydroxocobalamin 20 ng/ml; methylcobalamin 20 ng/ml; biotin 30 ng/ml; vitamin A 10 µg/ml; vitamin E 150 µg/ml; and ascorbate 100 µg/ml. Samples with no added vitamins were used as controls for all time intervals. Also red cells in saline were prepared by centri-

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fusing off the red cells from whole blood, washing the red cells three times with 0.9% NaCl (saline), and replacing the separated plasma with an equal volume of fresh saline. The red cell-saline preparations were then treated as whole blood.

Red blood cells in all procedures were obtained by centrifuging the plasma or saline to remove the buffy coat, and washing the red cells three times with saline. The vitamin content of the red cells was obtained by described methods (2); the B₁₂ level was obtained by using the protozoan *Ochromonas malhamensis*. Levels of nicotinates, B₆ vitamins, thiamin, and cobalamins were recorded as total activity of their respective congeners. The selective responses to folates of *Lactobacillus casei*, *Streptococcus faecalis*, and *Pediococcus cerevisiae* were used to differentiate various folates (2). *L. casei* responds to oxidized and reduced pteroylmonomono- to -triglutamates including N⁵-methyl-THF; *P. cerevisiae* responds to reduced pteroylmonomono- to -triglutamates excluding N⁵-methyl-THF; and *S. faecalis* responds to oxidized and reduced pteroylmonoglutamates but not to N⁵-methyl-THF. The *P. cerevisiae* value subtracted from the *S. faecalis* value gives the oxidized pteroylmonoglutamate value; subtracting oxidized and reduced pteroylmonoglutamate from the *L. casei* value yields oxidized pteroyltriglutamates plus any endogenous N⁵-methyl-THF content. In all instances, the *P. cerevisiae* values were divided by 2 because the racemic mixture (d,l-L⁵-formyltetrahydrofolate) was used for a standard, only the l-isomer is active.

L. casei is the only folate requirer that measures N⁵-methyl-THF, but cannot measure folates beyond the pteroyltriglutamate stage without prior folate deconjugation (2). Folylpolyglutamates in red cells were deconjugated (deglutamylated) by treatment with hog kidney conjugase to determine the folylpolyglutamate titer of the sample. Folylpolyglutamates were deconjugated to the monoglutamate stage by modifications of a described method (3). One milliliter of the sample was added to 4 ml of a solution containing 1.25% ascorbate and 0.5% NaCl at pH 6.0; the mixture was heated in boiling water for 10 min then cooled in an ice bath. After cooling, 5 ml of the ascorbate-NaCl solution was added to the homogenized red cell coagulum. After centrifuging off the debris, 6 ml of the supernatant was diluted with 6 ml of a powdered hog kidney suspension (0.4 mg/ml of powdered hog kidney suspended in 0.1 M acetate buffer, pH 4.7). The mixture was incubated overnight at 37 C, autoclaved 10 min, and assayed for various folates with the folate requirers described above (2).

Results

In vivo

With the exception of some folates, ascorbate, and vitamins A and E, the red cell vitamin titers increased after vitamin administration (Tables 1 and 2). The striking

TABLE 1
Means and standard deviations of vitamins in red cells after exposure to multivitamins in vivo and in vitro*

Vitamin	Concentration	In vivo			In vitro		
		Time, hr			Time, hr		
		0	3	24	0	3	24
A ^b	μg/100 ml	ND	ND	ND	ND	ND	ND
E ^b	mg/100 ml	0.09 (0.03)	0.09 (0.03)	0.09 (0.03)	0.09 (0.03)	0.09 (0.03)	0.09 (0.03)
Ascorbate	mg/100 ml	0.9 (0.31)	0.9 (0.35)	0.9 (0.32)	0.9 (0.30)	2.9 (1.1)	9.3 (3.1)
Nicotinic acid	μg/ml				8.6 (3.0)	12.9 (4.3)	25.0 (8.2)
Nicotinamide	μg/ml	7.1 (2.2)	8.8 (3.1)	8.0 (2.7)	8.9 (3.1)	10.8 (3.8)	11.1 (3.8)
Riboflavin	ng/ml	214 (74)	274 (94)	208 (71)	252 (85)	543 (180)	1,016 (342)
Pantoic acid	ng/ml	284 (97)	449 (153)	539 (170)	274 (92)	2,400 (416)	8,600 (2,970)
Thiamin (as HCl)	ng/ml	48 (16)	106 (34)	38 (15)	40 (12)	2,200 (740)	8,800 (2,975)
Thiamin (as TPD) ^c	ng/ml	39 (13)	262 (90)	55 (24)	37 (13)	24,300 (8,100)	33,000 (834)
Thiamin (as TPP) ^d	ng/ml	45 (15)	252 (83)	76 (33)	43 (15)	703 (240)	2,200 (863)
Biotin	pg/ml	89 (31)	218 (75)	89 (31)	132 (55)	5,875 (1,950)	9,333 (3,155)
Cyano cobalamin	pg/ml	55 (19)	81 (28)	56 (21)	59 (20)	450 (161)	1,005 (382)
Hydroxycobalamin	pg/ml	63 (22)	75 (25)	52 (28)	70 (23)	392 (134)	337 (115)
Methylcobalamin	pg/ml				64 (21)	150 (54)	417 (143)
Pyridoxal	ng/ml	24 (8)	598 (135)	35 (14)	35 (13)	1,672 (581)	6,625 (2,145)
Pyridoxal	ng/ml				29 (10)	9,861 (3,750)	11,268 (4,800)
Pyridoxamine	ng/ml				22 (9)	6,056 (2,060)	7,361 (2,410)
Pyridoxal-5-PO ₄	ng/ml				25 (17)	323 (110)	783 (264)

* In vivo vitamins given intravenously with exception of thiamin as TPD (thiamin propyldisulfide) given orally. Values in parentheses are standard deviations. ^b ND (Not detectable); sensitivity of method is 5 μg/100 ml. ^c Sensitivity of method is 0.09 mg/100 ml. ^d Thiamin pyrophosphate.

TABLE 2

Means and standard deviations, ng/ml, of folates in red cells after exposure to folates *in vivo* and *in vitro**

	Folate given								
	None	Folic acid		N ⁵ -formyl-THF		Pteroyltriglutamate		N ⁵ -methyl-THF	
	Time, hours								
	0	3	24	3	24	3	24	3	24
I. In vivo folate response									
N ⁵ -methyl-THF-monoglutamate	8.2 (3.1)	9.3 (3.2)	13 (4.4)	20 (7)	25 (9)	10.5 (3.7)	6.9 (2.8)		
N ⁵ -methyl-THF-polyglutamates	212 (75)	237 (94)	208 (70)	265 (92)	211 (87)	249 (85)	215 (73)		
Reduced THF-monoglutamates	1.1 (0.4)	1.0 (0.3)	0.8 (0.3)	1.2 (0.4)	1.7 (0.5)	1.0 (0.4)	0.5 (0.2)		
Reduced THF-polyglutamates	12 (4.0)	10 (3.4)	10 (3.1)	36 (12)	12 (3.8)	18 (6)	13 (3.5)		
Oxidized folyl-monoglutamate	0.6 (0.2)	0.5 (0.2)	0.6 (0.3)	0.4 (0.2)	1.6 (0.6)	0.5 (0.2)	0.4 (0.2)		
Oxidized folyl-polyglutamates	1.0 (0.4)	1.2 (0.5)	0.9 (0.4)	0.5 (0.2)	0.3 (0.2)	0.8 (0.3)	0.9 (0.4)		
II. In vitro folate response									
N ⁵ -methyl-THF-monoglutamate	5.6 (2.5)	8.5 (2.9)	27 (9)	63 (24)	145 (56)	32 (12)	18.7 (6.2)	27 (9.5)	8.0 (2.1)
N ⁵ -methyl-THF-polyglutamates	144 (52)	129 (44)	137 (46)	235 (80)	311 (93)	139 (48)	127 (33)	317 (108)	225 (77)
Reduced THF-monoglutamates	1.5 (0.5)	1.5 (0.6)	2.0 (0.8)	26 (9)	46 (14)	1.5 (0.5)	1.5 (0.4)	1.1 (0.4)	0.5 (0.2)
Reduced THF-polyglutamates	7.7 (2.6)	9.3 (2.7)	6.2 (2.1)	67 (23)	131 (45)	12.3 (4.1)	8.7 (3.0)	28 (8)	5.6 (2.2)
Oxidized folyl-monoglutamate	0.7 (0.2)	0.7 (0.3)	0.9 (0.4)	0.8 (0.4)	0.9 (0.5)	0.9 (0.3)	0.9 (0.4)	1.3 (0.5)	2.2 (0.8)
Oxidized folyl-polyglutamates	0.7 (0.3)	1.0 (0.4)	2.2 (0.7)	0.9 (0.3)	0.8 (0.3)	0.9 (0.4)	0.9 (0.4)	8.5 (2.9)	2.6 (0.9)

* Values in parentheses are standard deviations.

increases occurred with B₆, thiamin, and biotin; pantothenate levels rose even at 24 hr while other red cell vitamin levels were returning to almost the control titer. The thiamin HCl induced the least rise in red cell thiamin activity compared with thiamin pyrophosphate and orally administered thiamin propyldisulfide; the latter permitted markedly increased red cell thiamin levels and diffused into the cell more readily.

Hog kidney conjugase effectively deconjugated red cell folylpolyglutamates as judged from the increased values of the free folates after deconjugation (Table 2); the bulk of red cell folate activity was due to N⁵-methyl-THF polyglutamates, which is the predominant folate form found in red cells. Red cell N⁵-methyl-THF mono- and polygluta-

mates increased slightly 3 hr after administration of the folate vitamers. Other red cell-reduced folylpolyglutamates increased in 3 hr, but only after N⁵-formyl-THF was given; reduced folylmonoglutamates were unaffected. No folate vitamer increased any type of oxidized folates in red cells.

In vitro

Results were similar whether red cells were incubated in saline or plasma; therefore only plasma-red cell incubates are given in Tables 1 and 2. Since values for red cells incubated without added vitamins remained unchanged at all times, the unincubated sample was used as a control value.

All vitamins, except A and E, measurably entered the red cell during the experimental

interval; the longer the incubation the higher the vitamin titer. Higher red cell vitamin titers were present *in vitro* than *in vivo*. In contrast to the finding for the "in vivo" cells, ascorbate penetrated the red cell *in vitro*. The phosphorylated forms of the vitamin did not penetrate as well as their hydrochloride salts. Of all the analogs of B₆, thiamin, nicotinate, and B₁₂ (Table 1), pyridoxal HCl, thiamin propyldisulfide, nicotinic acid, and cyanocobalamin penetrated the red cell better and gave the highest activity when compared with their other analogs.

Incubation of red cells with the addition of N⁵-formyl-THF or N⁵-methyl-THF produced a sharp increase in mono- and polyglutamates of N⁵-methyl-THF as well as other reduced folates in red cells; incubation with N⁵-methyl-THF produced minimal increases in oxidized folates. Incubation of red cells with folic acid and Teropterin produced a slight increase in only N⁵-methyl-THF monoglutamate.

Discussion

Why some vitamins quickly enter red cells and others do not presumably depends on properties of scarcely explored transport mechanisms and diffusion effects. The results with ascorbate illustrate the puzzles: *in vivo* it does not penetrate the red cell; *in vitro* it does (Table 1). Perhaps other tissues quickly take up ascorbate at the expense of the red cell *in vivo*, whereas the vitamin accumulation *in vitro* permits the vitamin to diffuse into the cell; this may account for the higher vitamin titers seen when red cells are incubated *in vitro*. The higher vitamin avidity of storage depots such as liver and kidney may be a principal factor *in vivo*. Plasma is not a factor, either *in vivo* or *in vitro*; *in vivo* it does not bind vitamins at the expense of the red cell or other tissues (1). *In vitro* results with saline- or plasma-red cell incubates were the same, indicating that, here too, plasma did not bind vitamins at the expense of the red cell. Thus, plasma seems to be a vitamin transporter and does not seem to play a major role in influencing vitamin transfer into red cells. The mature red cell is not an active respiring tissue or *de novo* synthesizer of metabolically active compounds (4). It re-

tains some enzymes from the reticulocyte stage and can make some enzymes from the coenzyme precursors, e.g., nicotinamide adenine dinucleotide (NAD) from nicotinate and cocarboxylase from thiamin by adenosine triphosphate (ATP) phosphorylation. Our past study (1) shows that red cells also relinquish their retained excess exogenous vitamin load to more active tissues; this assumes that vitamin retention requires energy. Thus the mature red cell *in vivo*, like plasma, also seems to be a transporter of excess vitamins, in principle resembling hemoglobin, which transports oxygen by mass action.

The lipid-soluble vitamins A and E do not enter the red cell measurably either *in vitro* or *in vivo* (Table 1). As both vitamins are involved in stabilization of the lipoprotein membrane of red cells (5-7), they might be localized in the red cell membrane rather than within the cell (8, 9), thus allowing red cells to contain only slight spillage of these two vitamins from the membrane. As shown here and by others (1, 9, 10), there is no vitamin E entering the red cells even after the red cells are immersed in a vitamin E-enriched milieu. Vitamin E probably does not penetrate the red cell deeper than the lipid-rich membrane (9, 10); if so, it might have eluded detection in our assay system because we detected only intracellular rather than red cell membrane vitamin accumulation, i.e., in preparing samples for vitamin assay, the red cell stroma was centrifuged off after hemolysis and was discarded. Future work on vitamins in red cell membrane will take this into account.

The other vitamins entered the red cell *in vivo* and *in vitro*. As mentioned, the higher titers seen *in vitro* are probably due to the missing tissue vitamin depots that would have bound more vitamins, leaving fewer vitamins available for diffusion into red cells. This effect was further enhanced by incubating the cells with approximately 20 to 25 times more vitamins than are normally present in the circulation. The present results agree with other *in vitro* studies involving B₆ and B₁₂ transfer into red cells (11-13). As shown here, B₁₂ enters the red cell poorly *in vivo* when compared with *in vitro* incubation. Vitamin B₁₂ levels in red cells were lower than those previously reported (2). We attributed

this to the use of *O. malhamensis* for B_{12} assay; this organism is more specific for the metabolically active forms of B_{12} (2) and hence is not stimulated by other metabolites present in biologic fluids and tissues. All other vitamins offered the red cell seemed not to be mainly localized in the stroma (11) or, unlike vitamins A and E, they would not have shown intracellular increases in our assay system. In vitro, vitamin B_6 is rapidly taken up by the red cell (12); we have confirmed this in both systems. Pyridoxal-5-phosphate did not penetrate the cell in vitro as well as the other B_6 salts; thiamin pyrophosphate behaved similarly. Perhaps the electric charge on these vitamers hindered penetration or attachment to a transfer system. Phosphorylated thiamin is not absorbed through the gut as well as thiamin hydrochloride or thiamin propyldisulfide (14), indicating that even the intestinal cell, like the red cell, is not highly permeable for these phosphorylated vitamers. Perhaps cells prefer to do their own phosphorylations, as all forms of thiamin within the red cell and intestinal cells are phosphorylated via ATP (15, 16), as is glucose (17). This is a familiar situation: nucleotides are dephosphorylated and deribosylated to their free base at the cell membrane before penetration (18, 19).

Folates in red cells exist mainly as glutamyl derivatives of N^5 -methyl-THF (Table 2), and probably represent the principal depot form of folate in tissues (2). In vitro the red cell seems unable to reduce folates. Our results show that once the reduced folate N^5 -formyl-THF penetrates the red cell, a higher titer of N^5 -methyl-THF is produced; oxidized folates do not do this. Thus the red cell might methylate reduced folates more easily than the oxidized forms. N^5 -methyl-THF polyglutamates were also increased after incubation of red cells with N^5 -methyl-THF, indicating that red cells can form polyglutamyl folates. In vivo the liver converts oxidized folates into their reduced and methylated forms (2, 20, 21), thus producing the metabolically active forms of N^5 -methyl-THF (Table 2).

In vitro studies on rat intestine have shown that nicotinic acid, pantothenic acid, biotin, folate, and vitamin B_{12} can diffuse into in-

testinal cells (22); our in vitro studies show that a similar pattern exists for red cells. In vivo, many other factors such as ATP-mediated transport mechanisms (23, 24) and vitamin solubilizers (25, 26), e.g., bile, interplay in the promotion of cellular permeation.

The techniques described here could provide a simple, convenient model for preliminary screening of forms of vitamins designed for more efficient cell penetration and, as shown previously, body distribution (1).

Summary

The entry of vitamins in red cells after intravenous administration of multivitamins was determined in six healthy volunteers; as control, red cell samples from six volunteers were each incubated with vitamins. Except for some folates, ascorbate, vitamins A and E, all B-vitamin red cell titers increased after intravenous multivitamins. Oral administration of a lipid soluble ester of thiamin, thiamin propyldisulfide, penetrated the red cells better than intravenously administered thiamin hydrochloride or the pyrophosphate coenzyme. The most striking increases were seen with B_6 , thiamin, and biotin. N^5 -formyl-tetrahydrofolate increased reduced folylpolyglutamates but no folate vitamer could increase any type of oxidized folates in red cells.

After direct incubation, all vitamins, except for A and E, entered the red cell; in contrast to the results seen in vivo, ascorbate penetrated red cell suspensions. Phosphorylated thiamin and pyridoxal did not penetrate as well as their free bases. Thiamin propyldisulfide, nicotinic acid, pyridoxal hydrochloride, and cyanocobalamin entered the red cell better than other respective analogs. Red cells incubated with N^5 -formyl-tetrahydrofolate or N^5 -methyl-tetrahydrofolate showed large increments in the mono- and polyglutamates of N^5 -methyl-tetrahydrofolate. In vivo, most B-vitamins quickly concentrate in tissues and result in lower intracellular red cell vitamin titers than seen in vitro. In vitro, most vitamins seem to concentrate in red cells by diffusion and thus higher vitamin titers are attained in the cells with longer periods of incubation, whereas

in vivo the avidity of other vitamin depots decreased availability of vitamins for red cells.

We conclude that red cells, like plasma, are capable of transporting vitamins. ■

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INFLUENCE OF MASSIVE DOSES OF VITAMIN B₁ ON FERTILITY AND LACTATION¹

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Following the numerous reports on the toxic effects of massive doses of vitamin D, it was logical for nutritional investigators to be interested in the accumulation of evidence on the possible existence of other hypervitaminoses. For some years it was planned to study vitamin B₁ from this standpoint but our studies on the biochemistry and pathology of deficiency diseases did not permit following this line of research. The report, however, of Perla ('37) that vitamin B₁ in amounts equivalent to forty times the maintenance requirement produced toxic effects as evidenced by inability of rats to rear their young successfully, stimulated this investigation, which was begun in January of 1938.

Perla used a stock diet consisting of 15 gm. per day of a basic mixture of hominy 100 parts, rolled oats 15 parts, fine meat and bone 25 parts, salts 1½ parts, and dried milk 16 parts, to which was added a few drops of cod liver oil, 0.3 gm. of wheat germ and 0.3 gm. of crude Fleischman's brewer's yeast per rat. He found that supplementing his stock diet with Mead Johnson's brewer's yeast equivalent to 50 I.U. of vitamin B₁ per animal per day resulted in a disturbance in lactation in the first generation which was accentuated in the second generation. The effect was more pronounced in

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animals fed a vitamin B₁ concentrate (adsorbate) among which still-births were also common. Synthetic vitamin B₁ was somewhat less toxic than the vitamin B₁ adsorbate but similar interference with lactation occurred in the second generation.

While the work initiated in our laboratory was in progress the monograph on vitamin B₁ (thiamin)² by Williams and Spies ('38) appeared in which they criticized the findings of Perla. Since Perla injected the synthetic vitamin B₁ subcutaneously, they state: "The disturbances of very young litters for purposes of daily injections seems a questionable practice which might well lead to the abandonment of the young by the mothers." Furthermore, they refer to experiments of Ammerman and Waterman who administered 80 to 1000 µg. of thiamin daily to each rat as a supplement to a stock diet, which was continued for three generations, and reproduction was perfectly normal. Since no reference is given to this work in the bibliography, the work quoted probably represents unpublished data. Without knowing the composition of the stock diet and in the absence of experimental data, it is impossible to evaluate the investigations of Ammerman and Waterman. But at least it appears that we have conflicting results on the influence of massive doses of vitamin B₁ on lactation efficiency when carried on for several generations. It is therefore, all the more pertinent that the results of this laboratory be reported at this time.

Since 1920 we have employed the Wistar strain of albino rat and have successfully reared our animals on the following stock diet: whole wheat, 27; rolled oats, 26; yellow corn, 22; linseed meal, 15; commercial casein, 5; cod liver oil, 1; NaCl, 0.5; and CaCO₃, 0.5. This diet is supplemented 6 days a week with 5 cc. of fresh cow's milk per animal until breeding when it is increased to 10 cc. and during lactation, when the young begin eating, to 15 cc. daily. Each animal also receives 15 gm. fresh lettuce once weekly. On such a ration we raise 95 to 98% of our young, the mortality being less

² Vitamin B₁ and thiamin are used interchangeably in this paper.

than 5%, and very seldom do we encounter a case of sterility. When we attempted to remove either the supplementary lettuce or milk from this stock diet, the infant mortality increased to over 50%. Removing both the milk and lettuce increased the infant mortality to 60% accompanied by sterility. Therefore, as a starting point in this research, our stock diet supplemented with milk and lettuce was employed.

EXPERIMENTAL

The rats in this study were divided into four groups: A) each animal received 10 µg. thiamin daily; B) each animal received 100 µg. thiamin daily; C) each animal received 200 µg. thiamin daily, and D) each animal received 400 µg. thiamin daily. The thiamin used was the pure crystalline product of Merck.³ The results are summarized in table 1.

Group A. Since 10 µg. of thiamin was found sufficient as a curative dose for even the most marked cases of polyncuritis associated with convulsions and at the same time was accompanied by very good growth, it was at first thought to use this daily amount as a standard of comparison. This was hardly justifiable, since the stock diet furnished in itself liberal amounts of vitamin B₁. On this daily dose fertility and lactation was perfectly normal for two generations. Therefore, it was considered advisable to use a large dose during the third generation. The daily dose was increased to 200 µg. thiamin daily. Toxic effects then became apparent. The lactation efficiency dropped from 95 to 41%; also, one female was sterile and post-mortem examination showed resorption of twelve remaining embryos. The resorption was indistinguishable from vitamin E deficiency.

Group B. No toxic effects were encountered in the first generation on the 100 µg. thiamin daily dose. In the second generation, however, two females were sterile, but the two females that were fertile reared their young successfully. In the third generation when the daily thiamin dose was in-

³ We wish to express our appreciation to Merck and Co. for the thiamin donated for this work.

creased to 400 μ g., five females completely failed to rear their seven litters and one was sterile. In other words, infant mortality was 100%. Almost invariably the litters died the first few days of the nursing period and cannibalism was very marked, which was associated with a loss of the maternal instinct.

Group C. On the daily dose of 200 μ g. thiamin, two mothers out of six became sterile in the first generation. Since only

TABLE 1
Influence of massive doses of vitamin B₁ on fertility and lactation

Group	Vitamin B ₁ daily dose	Generation	Females	Litters	Young born	Young allowed to be reared	Young weaned	Per cent young weaned	Remarks
A	10 μ g.	First	4	4	37	24	23	96	One female was sterile after 161 days of mating. Another female resorbed twelve embryos.
	10 μ g.	Second	6	6	49	32	30	94	
	200 μ g.	Third	6	6	59	36	15	41	
B	100 μ g.	First	4	4	36	24	24	100	Two females were sterile. One female was sterile after 110 days of mating.
	160 μ g.	Second	4	2	21	12	12	100	
	400 μ g.	Third	6	7	64	42	0	0	
C	200 μ g.	First	6	4	43	24	24	100	Two females were sterile. Four females were sterile 120 days after mating. One female resorbed fourteen embryos.
	200 μ g.	Second	2	2	12	12	12	100	
	600 μ g.	Third	6	2	25	12	0	0	
D	400 μ g.	First	4	6	61	34	32	94	
	400 μ g.	Second	6	6	69	36	30	83	
	800 μ g.	Third	6	14	147	78	10	12	

two mothers were available for the second generation, the insufficient number of animals are not a criterion for the fertility and lactation performances. In the third generation on the 600 µg. daily thiamin dose, however, out of six females four were sterile, one with a clear case of resorption indistinguishable from vitamin E deficiency and the other two that were fertile entirely failed to rear their litters, death of the young having occurred during the first few days of lactation.

Group D. It is surprising that on the 400 µg. daily thiamin dose, fertility and lactation should have proceeded normally during the first two generations, but lactation was a pronounced failure during the third generation on the 800 µg. daily vitamin B₁ dose, the infant mortality being 88%.

Growth in all instances with the thiamin supplements was much greater than on the stock diet alone.

At no time throughout this study was the regular stock diet with its supplements of milk and lettuce changed, and all the daily thiamin administrations were given orally in solution in Petri dishes, which all the animals eagerly consumed. The mothers were tame at all times, although when by virtue of the large doses of thiamin they were unable to lactate, they became disinterested in the welfare of their young and devoured them, a phenomenon characteristic of the experimental rat when there is a deficiency of an essential factor in the diet.

While Perla⁴ on his stock diet began to encounter toxic symptoms on lactation in the first generation, the injurious effects on lactation of massive doses of thiamin, supplementing our stock diet were not manifested until the third generation. The injurious effects on fertility, however, are apparent in the first generation. Beginning with a 400 µg. thiamin daily dose the physiological mechanism of milk secretion

⁴Perla's recent findings ('39) that a daily allowance of 2 mg. manganous chloride counteracts the toxicity of large doses of thiamin lactation is of considerable interest. It would link vitamin B₁ metabolism with manganese, as vitamin D is associated with calcium and phosphorus metabolism. Also the giving of manganese salts might be indicated in various types of neuritis or other diseases where large doses of this vitamin are prescribed.

collapses. Out of 132 young allowed to be reared on 400 to 800 $\mu\text{g.}$ daily doses of thiamin, only ten young were reared. Since this work was completed, Perla ('39) in a preliminary report also cites progressive decrease in fertility on daily supplements of 30 I.U. of vitamin B_1 .

The fact that resorption of the foetus during gestation was observed in vitamin A deficiency (Sure, '28) and in toxic doses of vitamin B_1 , we can no longer consider such phenomenon as specific for vitamin E deficiency.

The question arises: What daily dose of thiamin did the stock diet with its supplements provide? Before such calculation can be made it is necessary to know the thiamin content of each of the constituents of this diet expressed as micrograms per gram. From the thiamin content of foodstuffs given by Williams and Spies in their monograph ('38) whole wheat, rolled oats and yellow corn contain 4.72, 3.00, and 2.5 $\mu\text{g.}$ per gram respectively. No figures are given for linseed meal nor for commercial casein. But it is estimated that linseed meal may contain 3 $\mu\text{g.}$ per gram and commercial casein 2.0 $\mu\text{g.}$ per gram. The latter should be a liberal figure, since milk powder contains only 2.5 $\mu\text{g.}$ per gram. Lettuce contains 0.2 $\mu\text{g.}$ per gram fresh tissue, and whole milk 0.45 $\mu\text{g.}$ per gram. From such figures it is estimated that 100 gm. of our stock diet will contain about 328 $\mu\text{g.}$ thiamin. A growing animal eating 15 gm. of such feed daily will then receive approximately 50 $\mu\text{g.}$ of thiamin. A lactating rat which is eating 25 to 30 gm. of feed and drinking 10 to 15 cc. of fresh cow's milk, will consume 100 to 110 $\mu\text{g.}$ thiamin daily. Since sterility was encountered in the second generation on 100 $\mu\text{g.}$ of thiamin supplementing our stock diet, which in itself provided an equal dose of this vitamin, it appears that twice the daily intake of thiamin furnished by our stock ration interferes with reproduction. Since 2.5 $\mu\text{g.}$ thiamin daily per rat will prevent polyneuritic symptoms and loss of weight, eighty times the maintenance dose of thiamin is toxic from the standpoint of reproduction and 120 times the maintenance dose definitely toxic for lactation. Furthermore, studies just

completed on lactation with purified diets (using crystalline thiamin, riboflavin, B₆, choline, nicotinic acid and W factor as components of the vitamin B complex) indicate the daily requirement of vitamin B₁ to be 120 μ g. In other words, three times the requirements for lactation (as found in short time transfer experiments from stock diet to synthetic diets) are definitely toxic in the third generation animals fed our stock diet. Just what manganese may do to counteract such toxicity (Perla, '39) is being checked at this time. When it is considered that Bills ('30) found that vitamin D (activated ergosterol) administered to rats in doses 100 times greater than the minimum antiricketic level did not produce a toxic effect on growth and reproduction, and that 1000 times the overdosage was just perceptibly harmful, and that it took a 4000 times overdosage to be definitely injurious, the possible toxic effects of vitamin B₁ or thiamin appear quite serious. It is doubtful, however, since vitamin B₁ is quite deficient in the American diet (Sure, '33; McCollum, '35) whether we will encounter injurious effects from an overdosage. However, we must bear in mind that large doses of thiamin for long periods of time may affect the pregnant and nursing mother.

SUMMARY

A daily dose of 100 μ g. of thiamin results in female sterility in the second generation. A daily dose of 200 μ g. of thiamin produces toxic effects in lactation in the third generation. A daily dose of 400 μ g. of thiamin results in entire failure in lactation in the third generation.

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MODIFICATION OF "B VITAMIN" CONTENT DURING EMBRYOLOGICAL DEVELOPMENT

By

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Free-living animals subsisting on an adequate diet appear to be unaffected by a moderately increased intake of vitamins of the "B complex." The addition of several times the daily requirement of pantothenic acid, thiamin, nicotinic acid or riboflavin has been reported to be without observable physiological effects in several types of animals (1, 2, 3, 4). Apparently the normal adult animal is equipped to take care of considerable excess of these necessary dietary components over and above the irreducible minimum which is so essential for normal function.

Most of the work in this respect has been carried out on animals which had attained the free-living status. Little if any attention has been given to the effect of producing this type of vitamin imbalance in the developing embryo. Investigations along these lines would appear to be especially worthwhile since there are so many factors such as rapid growth, morphological differentiation and special types of metabolism present in the early part of development which are not found in the post embryological period.

The present study was undertaken to discover what effect an increase of one of the vitamins (e.g., pantothenic acid, thiamin, riboflavin or nicotinic acid) in the egg has on the growth of certain organs and on the hemoglobin concentration of the chick embryo blood. Data were also obtained on certain other matters not foreseen at the commencement of the investigation.

The chick embryo is well suited for such a project since together with the egg it constitutes a relatively independent biological system in which temperature and food are stable factors. The main difficulty encountered was the introduction of the vitamins under investigation in such a manner as to eliminate effects other than those due to the vitamin itself.

MATERIAL AND METHODS

The eggs used were taken from a pure blooded flock of white leghorn hens whose diet was well balanced and apparently adequate in all respects. Control and experimental eggs were paired as to weight, time laid, and time of removal from the incubator.

The vitamin level of the eggs was raised by injection directly into the eggs before incubation, except that in one experiment with pantothenic acid the vitamin level of the eggs was raised by supplementing the diet of the hens. As a medium for injection sterile egg white proved fairly satisfactory, and the vitamins used dissolved readily in this fluid with the exception of riboflavin which dissolved only partly, the balance being present

as a suspension. The concentration of the vitamin in the injected egg white was adjusted so that it was necessary to introduce only 0.05 ml. per egg. The controls received the same dosage of pure egg white.

Injections were made in an area of the egg about midway between the equator and the small end. Here a space was cleaned with 70 per cent ethanol, and after this had evaporated a blunt probe was used to tap a tiny entrance through the shell, leaving the shell membrane intact. This operation required but a few seconds and only rarely was there any tendency for the remaining shell to fracture.

A tuberculin syringe of 1 ml. capacity fitted with an 18 gauge needle which had been shortened to about a centimeter in length was found to be satisfactory. A rubber washer was fitted over the needle so as to regulate the depth of penetration into the egg and prevent the possibility of back flow and loss of the injected material. The opening of the egg was sealed with transparent cellulose tape.

It was necessary to take special precautions against injuring the yolk membrane. This was accomplished by holding the egg in such a manner that the injection was made into the under surface. Since the yolk floats to the top it was rarely injured if this precaution were taken.

It has been shown by Snell, Aline, Couch, and Pearson (5) that the pantothenic acid level of the egg is proportional to the pantothenic acid level in the diet. They were able to show that supplementing the diet with about three times the daily requirement of this vitamin resulted in an increase in the egg to more than twice the normal level in a little over two weeks of the supplemented diet. In the present experiment the same procedure was followed for one phase of the investigation. The diet of a flock of hens was supplemented with about three times the normal requirement of pantothenic acid. Eggs from these hens were incubated in groups consisting of: group 1, eggs laid 4-6 days after the special feeding was initiated; group 2, eggs laid 7-9 days after initiating the supplemented diet; group 3, eggs laid 11-14 days after initiating supplemented diet; and group 4, eggs laid 1-3 days after the supplemented diet was discontinued. The control eggs for each of these groups differed only in the factor of increased concentration of pantothenic acid.

The eggs were generally incubated 11-13 days. At the end of this period they were removed from the incubator a few at a time, experimental and control eggs of the same weights. The embryos were removed and after obtaining blood from the omphalomesenteric arteries for hemoglobin determinations, they were weighed on analytical balances to milligram accuracy. The hemoglobin concentration of the blood was determined by the method described by Evelyn (6) using the Evelyn photoelectric colorimeter.

After being weighed, the embryos were placed in 95 per cent ethanol. Several changes of this medium hardened the tissues sufficiently for dissection. The heart, brain, and liver were carefully dissected out with the aid of a binocular dissecting microscope. The feet up to the junction with

the antebrachium were also removed. The dry weight of these organs was obtained and for purposes of comparison expressed in terms of percentage of the dry weight of the whole embryo.

In order to test the effect of the vitamins investigated on the development of malformities in the embryo, some eggs were injected with distilled water either by itself or mixed in small amounts with the egg white medium. The experimental eggs received in addition to this solution one of the vitamins under investigation. In these experiments the total amount of fluid injected did not exceed 0.05 ml. per egg.

In order to obtain data on the effect of increasing the level of one vitamin on the general vitamin level of the tissues 6 control and 6 experimental embryos from group 3 of the supplemented diet experiment were allowed to hatch. A composite of liver, brain, and heart tissues from each of these groups of chicks was analyzed for pantothenic acid, inositol, nicotinic acid, riboflavin, "folic acid," thiamin, pyridoxin, and biotin.

RESULTS

Tables 14, 15, 16, and 17 summarize the data obtained on the effect of a raised level in the egg of the vitamins studied on the hemoglobin concentration of chick embryo blood. Riboflavin and nicotinic acid at the levels reported were without definite results in this regard. An increased thiamin level in the egg was associated with a rise in the hemoglobin content of the embryo blood (Table 15). The most striking effect was induced in the embryos from eggs which had received the thiamin injection four days after incubation was initiated.

Pantothenic acid injected into the egg before incubation also effected an increase in the hemoglobin of the chick embryo blood (Table 14). The embryos from eggs produced by the flock on a diet supplemented with pantothenic acid manifested this trend more than the embryos from the eggs receiving extra pantothenic acid by injection.

The results also indicate that increasing the vitamin level of the egg may change to some extent the relative size of certain organs. However these changes were not of a degree to be considered in the class of abnormalities. These data are summarized in Tables 18 and 19. At the levels injected into the egg all four of the vitamins investigated induced

TABLE 14

The Effect of an Increase of Pantothenic Acid in the Egg on the Hemoglobin Content of the Blood of the Chick Embryo

The level in the egg raised 10 to 20 per cent by injection before incubation

Experiment	No. of Embryos	Incubation Time Days	Mean Hemoglobin Gm./100 ml.	S.D.	P.E. Diff. Between Means	$\frac{D}{P.E.}$	Hemoglobin Per cent Control = 100
1. Control _____	11	12-13	4.32	0.74	—	—	—
1. P. A. _____	11	12-13	4.89	1.00	0.069	8.2	113
2. Control _____	17	12-13	4.99	1.29	—	—	—
2. P. A. _____	17	12-13	5.33	1.20	0.064	5.3	107
3. Control _____	14	12-13	4.63	0.93	—	—	—
3. P. A. _____	14	12-13	4.95	0.86	0.062	5.3	107
Total Average:							
Control _____	42	12-13	4.65	0.98	—	—	—
P. A. _____	42	12-13	5.06	1.02	0.045	9.1	108

Vitamin level of eggs raised by supplementing diet of hens with three times daily requirement of pantothenic acid

Group 1. Control _____	10	12-13	4.92	0.65	—	—	—
Group 1. P. A.* _____	10	12-13	5.72	1.11	0.099	8.1	116
Group 2. Control _____	12	12-13	4.24	0.86	—	—	—
Group 2. P. A. _____	12	11-12	4.77	0.71	0.074	7.2	113
Group 3. Control _____	8	12-13	4.30	1.06	—	—	—
Group 3. P. A. _____	8	12-13	4.65	0.61	0.090	3.9	108
Group 4. Control _____	13	11-12	3.65	0.87	—	—	—
Group 4. P. A. _____	13	11-12	4.35	0.83	0.064	9.2	119
Total Average:							
Control _____	43	11-13	4.28	0.86	—	—	—
P. A. _____	43	11-13	4.87	0.82	0.063	9.3	114

*Group 1, eggs collected 4-6 days after initiating supplemented diet; group 2, 7-9 days; group 3, 11-14 days; group 4, 1-3 days after discontinuing supplemented diet.

"B Vitamin" Content During Embryological Development

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TABLE 15

The Effect of an Increase of the Thiamin in the Egg on the Hemoglobin Content of the Blood of the Chick Embryo

The level in the eggs raised 10 to 20 per cent by injection before incubation

Experiment	No. of Embryos	Incubation Time Days	Mean Hemoglobin Gm./100 ml.	S.D.	P.E. Diff. Between Means	D P.E.	Hemoglobin Per cent Control = 100
1. Control	12	-----	3.33	0.80	-----	-----	-----
1. Thiamin	12	11-13	3.36	0.79	0.068	0.44	101
2. Control	13	-----	3.24	0.83	-----	-----	-----
2. Thiamin	13	11-13	3.48	1.41	0.101	2.40	107
3. Control	15	-----	5.18	0.90	-----	-----	-----
3. Thiamin	15	12-13	5.71	0.48	0.055	9.67	110
4. Control	15	-----	4.63	0.89	-----	-----	-----
4. Thiamin	15	12-13	4.87	0.79	0.069	3.48	105
5. Control	10	-----	4.16	0.54	-----	-----	-----
5. Thiamin*	10	11-12	5.18	0.73	0.048	21.30	125
Total Average:							
Control	65	11-13	4.11	0.79	-----	-----	-----
Thiamin	65	11-13	4.52	0.84	0.063	6.5	110

*In this instance thiamin was injected four days after incubation began.

TABLE 16

The Effect of an Increase of Riboflavin in the Egg on the Hemoglobin Content of the Blood of the Chick Embryo

The level in the eggs raised 10 to 20 per cent by injection before incubation

Experiment	No. of Embryos	Incubation Time Days	Mean Hemoglobin Gm./100 ml.	S.D.	P.E. Diff. Between Means	D P.E.	Hemoglobin Per cent Control = 100
1. Control	18	-----	4.47	1.34	-----	-----	-----
1. Riboflavin	18	11-12	4.44	1.03	0.071	0.42	99
2. Control	15	-----	4.63	0.89	-----	-----	-----
2. Riboflavin	15	12-13	4.65	0.79	0.041	0.50	100
Total Average:							
Control	33	11-13	4.55	1.12	-----	-----	-----
Riboflavin	33	11-13	4.54	0.91	0.019	0.50	100

TABLE 17

The Effect of an Increase of Nicotinic Acid in the Egg on the Hemoglobin Content of the Blood of the Chick Embryo

The level in the egg raised 10 to 20 per cent by injection before incubation

Experiment	No. of Embryos	Incubation Time Days	Mean Hemoglobin Gm./100 ml.	S.D.	P.E. Diff. Between Means	D P.E.	Hemoglobin Per cent Control = 100
1. Control	6	-----	4.97	0.66	-----	-----	-----
1. N. A.	6	13-14	4.48	0.47	0.132	3.71	90
2. Control	19	-----	4.59	1.07	-----	-----	-----
2. N. A.	19	11-12	4.55	1.20	0.055	0.73	99
3. Control	19	-----	4.02	0.94	-----	-----	-----
3. N. A.	19	12-13	4.05	0.78	0.042	0.71	101
4. Control	18	-----	4.05	0.95	-----	-----	-----
4. N. A.	18	12-13	4.18	0.91	0.053	2.45	103
Total Average:							
Control	62	11-14	4.41	0.91	-----	-----	-----
N. A.	62	11-14	4.32	0.84	0.074	1.2	98

an increase in the relative brain weight of the embryos. However, in the group of eggs from the hens on the diet supplemented with pantothenic acid the relative brain weights of the embryos differed according to the time the diet had been in effect.

The embryos of groups 1 and 2 had brains relatively larger than the controls, but groups 3 and 4 manifested an opposite trend. The heart was increased in size in the embryos from eggs injected with thiamin and nicotinic acid, while injected pantothenic acid and riboflavin tended to depress the relative heart weight in comparison to the controls. As with the brain, so the embryonic hearts from eggs produced by the hens on a diet supplemented with pantothenic acid were much depressed in relative size down to 18 per cent below the controls.

In all the experiments completed the effect on the relative weight of the liver was variable and inconsistent. The feet in general tended to be relatively smaller in the experimental animals, but with exception of the embryos from the thiamin injected eggs the results were not clear cut.

Table 20 summarizes the results obtained on the effect of the experimental procedure on body weight. These data disclose that this aspect of the embryo was practically unaffected at the vitamin levels used.

Embryo survival was affected in the pantothenic acid supplemented diet experiment (Table 21). It will be seen that embryo viability was definitely better in the experimental eggs and reached the climax in the group which were laid when the supplemented diet had been in effect the longest period of time.

The effect of the vitamin level in the egg on the development of deformities is described in Table 22. It will be noted that when a medium such as distilled water was injected into the egg thus inducing malformities in the embryo, the presence of one of the vitamins used in this investigation materially increased the incidence of embryo deformity. Pantothenic acid and thiamin were much more potent in this respect than riboflavin and nicotinic acid at least at the levels used.

Table 23 summarizes the data obtained on the effect of increasing the level of one vitamin on the vitamin level of the tissues. It will be noted that raising the level of pantothenic acid in the egg by placing the hens on a supplemented diet tended to change very little the concentration of this vitamin in the liver and brain, but the experimental hearts contained 20 per cent more pantothenic acid than the hearts of the controls. In association with increased pantothenic acid in the egg the concentration of some of the other vitamins appeared to be affected. Inositol, nicotinic acid, riboflavin, "folic acid," thiamin, pyridoxin, and biotin were all near normal in concentration in the heart muscle, but the concentration of all the vitamins considered was lower in the liver of the experimental chicks with the exception of inositol and biotin. In the brain all tended to be lower than normal except pantothenic acid and pyridoxin.

It must be added that the results given in Table 23 are tentative in nature and may be revised as further studies are made in this field.

TABLE 18

The Effect of Increased Vitamin Concentration in the Egg on the Growth of the Brain, Heart, Liver, and Feet of the Chick Embryo

Vitamin level in the egg raised by injection before incubation

No. of Embryos	Age Days	Brain to Embryo Dry Wts. Per cent	Relative Brain Size C = 100	Heart to Embryo Dry Wts. Per cent	Relative Heart Size C = 100	Liver to Embryo Dry Wts. Per cent	Relative Liver Size C = 100	Feet to Embryo Dry Wts. Per cent	Relative Feet Size C = 100
Control—61	11-13	6.97 ± 0.10	—	1.19 ± 0.03	—	3.85 ± 0.09	—	3.31 ± 0.02	—
Thiamin*—58	11-13	7.60 ± 0.19	109	1.20 ± 0.01	101	3.62 ± 0.04	94	2.90 ± 0.04	88
Control—38	11-12	4.80 ± 0.25	—	1.22 ± 0.01	—	3.75 ± 0.13	—	3.11 ± 0.17	—
Pantothenic Acid—26	11-12	5.14 ± 0.29	107	1.13 ± 0.04	93	3.79 ± 0.27	101	3.01 ± 0.24	97
Control—89	11-13	5.12 ± 0.04	—	1.24 ± 0.05	—	3.98 ± 0.04	—	3.08 ± 0.26	—
Riboflavin—45	11-13	6.04 ± 0.45	118	1.15 ± 0.04	93	3.88 ± 0.07	98	3.01 ± 0.32	98
Control—89	12-14	4.58 ± 0.05	—	1.11 ± 0.14	—	3.70 ± 0.50	—	2.84 ± 0.08	—
Nicotinic Acid—78	12-14	4.75 ± 0.04	104	1.12 ± 0.08	101	3.77 ± 0.63	102	3.05 ± 0.04	107

*Thiamin level in the egg raised 15-50 per cent; pantothenic acid, 10-20 per cent; riboflavin, 10-30 per cent; nicotinic acid, 10-30 per cent.

TABLE 19

The Effect of Increased Pantothenic Acid in the Egg on the Growth of the Brain, Heart, Liver, and Feet of the Chick Embryo

Vitamin level of eggs raised by supplementing diet of hens with three times daily requirement of pantothenic acid

Experiment	No. of Embryos	Age Days	Brain to Embryo Dry Wts. Per cent	Relative Brain Size C = 100	Heart to Embryo Dry Wts. Per cent	Relative Heart Size C = 100	Liver to Embryo Dry Wts. Per cent	Relative Liver Size C = 100	Feet to Embryo Dry Wts. Per cent	Relative Feet Size C = 100
Group 1—Control	12	13-14	3.68	—	0.95	—	2.94	—	2.93	—
Group 1—P.A.*	29	13-14	3.90	106	0.95	100	2.98	102	2.98	102
Group 2—Control	13	11-12	5.50	—	1.42	—	1.91	—	1.91	—
Group 2—P. A.	30	11-12	5.65	103	1.16	82	1.71	90	1.71	90
Group 3—Control	8	12-13	4.98	—	1.39	—	3.02	—	3.02	—
Group 3—P. A.	24	12-13	4.77	96	1.15	83	3.06	101	3.06	102
Group 4—Control	13	11-12	5.40	—	1.39	—	3.05	—	3.05	—
Group 4—P. A.	27	11-12	4.87	90	1.18	85	2.76	91	2.78	91

*Group 1, eggs collected 4-6 days after initiating supplemented diet; group 2, 7-9 days; group 3, 11-14 days; group 4, 1-3 days after discontinuing supplemented diet.

TABLE 20

The Effect of Increased Thiamin, Pantothenic Acid, Riboflavin, and Nicotinic Acid in the Egg of the Weight of the Chick Embryo

Experiment	No. of Embryos	Per cent Increase of Vitamin	Age Days	Mean Egg Weight Before Incubation Gm.	Mean Embryo Weight Gm.	Embryo in Per cent of Egg Weight	Relative Embryo Weight C = 100
Control	101	—	10-13	57.7	5.25 ± 0.45	9.09	—
Thiamin	102	10-50	10-13	58.0	5.34 ± 0.45	9.20	101
Control	79	—	11-13	58.7	4.90 ± 0.42	8.35	—
Pantothenic Acid	72	10-20	11-13	58.5	4.99 ± 0.42	8.52	102
Control	103	—	11-13	58.1	5.14 ± 0.35	8.85	—
Riboflavin	99	10-50	11-13	58.7	5.28 ± 0.44	9.01	102
Control	129	—	11-13	59.4	6.34 ± 0.56	10.68	—
Nicotinic Acid	117	10-30	11-13	59.3	6.21 ± 0.52	10.46	98

TABLE 21

The Effect of Increased Pantothenic Acid in the Egg on Embryo Survival

Pantothenic acid level of eggs raised by supplementing diet of hens with three times daily requirement of pantothenic acid

Experiment	Number Eggs	Number Live Embryos	Age Days	Per cent of live Embryos	Relative Viability Control = 100
Group 1. Control	18	18	12-13	72.2	—
Group 1. Pantothenic Acid*	86	80	12-13	88.3	115
Group 2. Control	18	18	11-12	72.2	—
Group 2. Pantothenic Acid	85	81	11-12	88.6	123
Group 3. Control	25	14	12-13	56.0	—
Group 3. Pantothenic Acid	48	35	12-13	72.9	130
Group 4. Control	19	13	11-12	68.4	—
Group 4. Pantothenic Acid	35	26	11-12	74.3	109
Total Average:					
Control	80	58	11-13	66.3	—
Pantothenic Acid	154	122	11-13	79.2	120

*Group 1, eggs collected 4-6 days after initiating supplemented diet; group 2, 7-9 days; group 3, 11-14 days; group 4, 1-3 days after discontinuing supplemented diet.

TABLE 22

The Effect of Increased Vitamin Concentration on the Incidence of Deformed Embryos Where Conditions in Both Experimental and Control Eggs Favor This Development

Vitamin level in egg raised 10 to 30 per cent by injection before incubation

Experiment	No. of Embryos	Age Days	Percentage Deformed	Relative Frequency of Deformity Control = 100
Control	108	11-13	10.2	—
Thiamin	141	11-13	28.2	277
Control	125	11-13	8.7	—
Pantothenic Acid	187	11-13	32.6	375
Control	103	11-13	7.3	—
Riboflavin	155	11-13	11.5	158
Control	106	12-14	8.2	—
Nicotinic Acid	143	12-14	12.6	154

TABLE 23

Effect of an Increase of Pantothenic Acid in the Egg on the Vitamin Level of the Liver, Brain, and Heart of the Day Old Chick

Vitamin level of eggs raised by supplementing diet of hens

Vitamin	Exper. Liver γ/gm.	Control Liver γ/gm.	Exper. Liver When Control = 100	Exper. Brain γ/gm.	Control Brain γ/gm.	Exper. Brain When Control = 100	Exper. Heart γ/gm.	Control Heart γ/gm.	Exper. Heart When Control = 100
Pantothenic									
Acid —	98.0	99.0	99.0	270.0	250.0	108.0	224.0	186.0	120
Inositol —	2600.0	2300.0	118.0	8800.0	13000.0	67.5	2700.0	2400.0	113
Nicotinic									
Acid —	260.0	410.0	63.5	200.0	230.0	87.0	262.0	246.0	107
Riboflavin	40.0	51.0	78.5	6.8	6.9	91.2	32.0	34.0	94
"Folic									
Acid" —	99.0	180.0	55.0	5.0	7.5	66.7	6.6	6.0	110
Thiamin —	5.2	5.7	91.0	2.5	5.3	47.0	2.8	2.9	97
Pyridoxin	1.4	2.4	59.2	2.5	1.3	192.0	0.98	0.77	127
Biotin —	0.50	0.39	128.0	0.028	0.038	74.0	0.078	0.075	104

DISCUSSION

It is evident from the results obtained that the chick embryo is susceptible to vitamin imbalances produced by increasing one of these food entities in the egg above the normal level. This fact was just as emphatically affirmed by the supplemented diet experiment as by the injection studies. But why the embryos reacted as they did to the procedures used is difficult to interpret. In the first place, the specific functions in biological processes of the vitamins used are still largely unknown, and in the second place, the metabolism of the chick embryo itself remains a fruitful field for investigation.

The effect of thiamin and pantothenic acid on the hemoglobin concentration of the chick embryo blood was one of the most consistent and statistically valid of all the results obtained. But aside from the fact that the hemoglobin is concerned with respiration even the part this compound plays in the chick embryo metabolism is unknown. It is common knowledge that the embryo in its early stages has a low blood hemoglobin concentration which rises with the age of the embryo to a high level at the time of hatching. Hemoglobin first appears about 33 hours after the beginning of incubation and thereafter steadily increases in concentration (7). When the embryo is very small it acquires part of its oxygen by other means than hemoglobin. Possibly the relatively high concentration of riboflavin in the white of the egg may serve to some degree as an oxygen carrier for the embryo. In the normal white there is enough of this vitamin present to give it a greenish cast, and it has been definitely established that riboflavin in addition to its function in

the oxidation-reduction cycle of the tissues may also serve in a capacity analogous to that of hemoglobin (8, 9). For the first 33 hours at least the rapidly growing embryo is able to satisfy its high respiratory rate without any hemoglobin whatever and even at the 11-13 day period, which marked the termination of incubation in the present study, the hemoglobin concentration is less than half of the post embryological level.

It is possible that a raised concentration of thiamin or pantothenic acid may interfere with the extra-hemoglobin sources of oxygen whatever they may be. In one experiment where the tissues of the embryo were analyzed for vitamin content after the level of the egg pantothenic acid had been raised 200 per cent by injection before incubation, there appeared to be a tendency for the riboflavin to be depressed. If this vitamin does serve in a manner similar to hemoglobin in early embryonic development, then its depression would necessitate an increase in the blood hemoglobin concentration.

If the respiratory rate of the embryo as a whole was raised, thus necessitating the transport of more oxygen to the tissues, a rise in the concentration of the blood hemoglobin would also be stimulated. It is possible that thiamin mediates its effect on hemoglobin concentration in this manner, since this vitamin has been definitely linked to carbohydrate metabolism and to biological oxidative mechanisms (1).

Riboflavin injected into the eggs before incubation did not affect the hemoglobin concentration of the embryo blood, and in view of its known capacity for action as an oxygen transporter mentioned above, it would not be expected to do so unless its dominant function in the embryo tended to offset this tendency.

A recent investigation has shown that the chick embryo is able to synthesize nicotinic acid and the total amount in the egg increases many fold during the course of development (10). Just what this means in relation to its place in embryo metabolism is unknown. Moderate increases in the egg level of this vitamin appear to be ineffective as far as hemoglobin concentration of the blood is concerned.

Warburg (11) has demonstrated the fact that embryonic tissues tend to differ markedly from the tissues of post embryonic animals in respect to respiratory rate and the degree of glycolysis. He showed further that within the embryo itself different organs may have dissimilar types of metabolism. The brain for example has a metabolism which is largely glycolytic in nature, and the respiratory rate of this organ is, in the early stages of development, much higher than other parts of the embryo (12). The sensitivity of this embryonic organ is illustrated by the tendency it manifested to become deformed when conditions in the egg were unfavorable in some respect. When abnormalities developed, the brain and eye were much more frequently affected than other embryonic organs and tissues. Further, if deformities developed in some other organ of the embryo and the brain remained normal morphologically, it was still affected to the extent of being smaller than normal.

In the comparatively low levels used in the injection experiments, the relative brain weight was increased in response to all four of the vitamins used. Nicotinic acid had the least effect in this regard, while riboflavin was most effective.

It is noteworthy that in the feeding experiments the effect of pantothenic acid on relative brain size depended on the level of concentration of this vitamin in the egg. At low levels there was a tendency for the brain to be larger than normal in agreement with the injection results, but at higher levels the relative brain size was below the control level. Whether comparatively high levels of thiamin, riboflavin, or nicotinic acid would also lead to a small brain size remains for further work to decide.

The effect on the relative brain size was in contrast to the fact that the total embryo weight was practically unaffected by all four of the vitamins at the levels given.

Raising the level of thiamin or nicotinic acid in the egg appeared to be without effect on the heart. Riboflavin and pantothenic acid both tended to depress the relative size of this organ. When the concentration of pantothenic acid in the egg was relatively high this tendency for a smaller embryonic heart was more pronounced.

The embryonic heart is unique among the organs present during early development in at least one respect. It is the only organ which at that time is regularly engaged in mechanical work. It is possible that its efficiency was increased by the above-normal concentration of pantothenic acid in the egg, making it unnecessary for it to occupy as large a percentage of the body as in the controls. In this connection, it was interesting to find that the higher concentration of pantothenic acid in the egg led to a slightly higher concentration of this vitamin in the heart muscle.

The effect of the vitamin imbalance on the embryo liver was probably of little significance. There was no consistent reaction on the part of this organ to the vitamin levels used. If anything, the tendency was for the liver to be relatively smaller in size. When toxic substances such as hypotonic fluid are introduced into the egg, the liver tends to become relatively much larger. Whenever conditions in the egg favored the development of deformed embryos, the liver was increased in size. This was probably due to the fact that the embryonic liver like the post embryonic liver plays a protective, detoxifying rôle in the organism.

It is not clear why the chick livers from the supplemented diet experiment showed no increase in pantothenic acid. As already pointed out, the tendency was for several other "B vitamins" to be even lower than normal in the liver tissue.

The feet were included in the list of the embryo parts considered in relation to raised vitamin levels in the egg because they are composed of a set of tissues entirely different from those involved in the brain, heart and liver. Also, they represent a part of the embryo which has a relatively low respiratory rate.

The effect of the vitamins used on the relative size of the feet was variable. Pantothenic acid and riboflavin were without much influence in this regard. Thiamin tended to reduce the size of the feet and nicotinic acid had just the opposite effect. It is possible that if the incubation time had been prolonged the size of the feet would have been affected to a greater degree. These appendages, unlike the brain, grow slowly in the early period of development.

Under optimum conditions and with eggs from a healthy vigorous flock of hens, there is little tendency for malformed embryos to develop. The eggs used in the present investigation normally produced less than one per cent of easily recognized deformities. The experimental procedures used, such as the injection into the egg of 0.05 ml. of sterile egg white before incubation, did not increase the incidence of embryo deformity as compared with untreated eggs.

If medium for injection is used which has a disturbing effect on the egg organization, many more malformed embryos develop than would normally be expected. Such fluids as olive oil, distilled water, infected egg white, and hypo- or hypertonic saline solutions when injected into the egg even in minute quantities will be associated with a sharp rise in embryo deformities.

The effect of injecting a raised level of one of the vitamins into the egg in one of these toxic media was to very definitely increase the incidence of deformity among the embryos. Thiamin and pantothenic acid were especially potent in this regard, increasing the number of defective embryos in the experimental groups two or three times above the controls. Not only was there a more widespread tendency in this regard in the pantothenic acid and thiamin treated eggs, but the degree of deformity tended to be more severe. Many of the embryos under these conditions became completely disorganized so that the usual plan and pattern was practically absent, while others were affected to a lesser degree.

As would be expected, the anterior end of the embryo was the most susceptible area. Defective eye and brain development were common under these circumstances. Some embryos were without one or both eyes and only the vestigial remains of a brain were present. No malformities were observed in the legs, wings, or musculature of the body. Embryos manifesting the highest degree of deformity lived only a short time. Most of these were dead by the end of the fifth day of development. Many embryos, however, with extreme head deformities lived to hatching time.

It would seem that after the embryo has been formed, which requires 72 hours in the chick, these deformities would not be likely to develop. However, it was found that even after the fifth day of incubation the injection of a hypotonic solution in the controls and this medium plus thiamin or pantothenic acid in the experimental eggs would stimulate the formation of abnormalities, the experimental eggs having a much higher incidence than the controls.

Where other factors were controlled, none of the vitamins used at the reported levels were associated with this tendency for deformed chicks to be developed. Under these conditions the eggs with the raised level of pantothenic acid in the supplemented diet experiment produced a higher percentage of live embryos than the controls in every group from the low to the high level. In group 3 of this experiment an electrical storm caused the current to the incubator to be shut off for many hours at about the fifth day of incubation, a critical time in chick embryo development. As the data given show, the experimental eggs produced 30 per cent more live embryos than the controls from this group. This was the highest differential in survival recorded. These results indicate that pantothenic acid supplemented to the diet of the hens definitely increased the hatchability of the eggs.

SUMMARY

1. The level of thiamin, pantothenic acid, riboflavin or nicotinic acid of the egg was raised 30 per cent or less by injection before incubation. This affected the 11 to 18 day chick embryo as follows:
 - a. Thiamin and pantothenic acid increased the blood hemoglobin concentration 10 per cent and 8 per cent respectively. Riboflavin and nicotinic acid were ineffective in this respect.
 - b. All four vitamins were associated with an increase in the relative size of the brain: thiamin 9 per cent, pantothenic acid 7 per cent, riboflavin 18 per cent, and nicotinic acid 4 per cent.
 - c. The effect on the relative size of the heart was variable. Thiamin and nicotinic acid had practically no effect, while pantothenic acid and riboflavin each depressed the relative size of this organ 7 per cent.
 - d. The relative size of the liver was not significantly affected.
 - e. The relative size of the feet was only slightly affected by pantothenic acid and riboflavin, but the thiamin group effected a 12 per cent decrease and the nicotinic acid group a 7 per cent increase.
2. Hens were placed on a pantothenic acid supplemented diet to raise the level of this vitamin in the eggs produced (5). Groups of eggs were obtained with vitamin levels varying according to the time the hens had been on the diet when the eggs were gathered. This procedure affected the embryo as follows:
 - a. Group 1—consisting of eggs collected 4–6 days after initiating the supplemented diet—produced embryos with a +16 per cent blood hemoglobin concentration, +6 per cent relative brain size, unaffected relative heart size. In all groups the effect on the relative size of the liver and feet was variable and of doubtful significance.
 - b. Group 2—eggs collected 7–9 days after initiating supplemented diet—blood hemoglobin concentration +13 per cent, relative brain size +3 per cent, relative heart size —18 per cent.

- c. Group 3—eggs collected 11–14 days after initiating the supplemented diet—blood hemoglobin concentration + 8 per cent, relative brain size, — 4 per cent, relative heart size — 17 per cent.
- d. Group 4—eggs from the period 1–3 days after discontinuing supplemented diet—blood hemoglobin concentration + 19 per cent, relative brain size — 10 per cent, relative heart size — 15 per cent.
3. The total weight of the embryos was practically unaffected by each of the four vitamins used.
4. The pantothenic acid supplemented diet experiment gave an average of 20 per cent better embryo survival than the controls, indicating a marked improvement in hatchability.
5. Injection of each of the four of the vitamins used increased the incidence of embryo deformity only when their effects were superimposed upon those of substances (e.g. distilled water) which tend to produce this type of development. Using an index of 100 to represent this deformity tendency in the controls, thiamin was 277, pantothenic acid 375, riboflavin 158, and nicotinic acid 154. These vitamins at the levels used, were not associated with disorganized types of growth in normal eggs.
6. Increasing the level of pantothenic acid in the eggs by supplemented feeding did not change the level of this vitamin in the liver and the brain of the day old chick. The level in the heart showed a tendency to rise. With an index of 100 representing the level of each vitamin in the control heart, liver, and brain, the level in the experimental heart, liver, and brain was:
 - heart—pantothenic acid 120, inositol 113, nicotinic acid 107, riboflavin 94, "folic acid" 110, thiamin 97, pyridoxin 127, and biotin 104.
 - liver—pantothenic acid 99, inositol 118, nicotinic acid 68.5, riboflavin 78.5, "folic acid" 55, thiamin 91, pyridoxin 59.2, and biotin 128.
 - brain—pantothenic acid 108, inositol 67.5, nicotinic acid 87, riboflavin 91.2, "folic acid" 66.7, thiamin 47, pyridoxin 192, and biotin 74.

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EFFECT OF B VITAMINS IN THE DIET ON TUMOR TRANSPLANTS

By

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Considerable attention has been given to the problem of the role of vitamins in the origin and growth of cancer. Most of the investigations in this field, however, have been handicapped, since it is only recently that many of these nutrilities have been available in a pure form. Further, until the present, even such basic information as data on the levels of the vitamins in the various tissues of different animals was lacking. This was especially true of the B vitamins, the importance of which has been emphasized by the fact that these vitamins are required for the normal functioning of all or nearly all living cells.

The present and preceding collection of papers has made available for the first time not only extensive data on the concentration of the various B vitamins in normal tissues but also on the levels of these entities in neoplastic tissue (1). Hence, it is now possible to approach the problem of the role of the vitamins in cancer tissue in a systematic manner.

The present study was concerned with the effect of various levels of some of the B vitamins on the susceptibility of the mouse to tumor transplants.

MATERIALS AND METHODS

A total of 140 dba mice composed equally of males and females and of about the same age and weight were used in this investigation. The animals were divided into 7 groups of 20 each. They were placed five to a cage, and such conditions as light, temperature and aeration were the same for all the mice concerned.

The mice of group I served as controls and were maintained on a diet of Purina dog chow—hereafter referred to as Purina. The B vitamin content of this food was determined and the results are recorded in Table I.

The other groups constituted the experimental animals and were given diets of varying vitamin content, the details of which are given in Table I. Group II was fed a diet of Purina supplemented with several times the amount of riboflavin, thiamin, nicotinic acid, pantothenic acid, inositol, and pyridoxin originally present in the ration. Choline was also added to what was estimated to be the same elevation as the B vitamins, but since no assay of Purina with respect to this substance was available, the figure in this instance is an approximation. Group III was maintained on a diet identical with that used for Group II except for the addition of 2% yeast extract. Group IV was given a diet of Purina plus addition of about 4% by weight of beef spleen extract. Group V was given a diet of Purina plus several times the amount of nicotinic acid, riboflavin, and pantothenic

TABLE I

Vitamin Content of Various Diets Used
γ/gm.

	Thiamin	Ribo- savin	Nicotinic Acid	Panto- themic Acid	Pyridoxin	Biotin	Inositol	Folic Acid*	Choline
Group I Purina (Control) _____	3.7	5.7	39	14	2.1	0.19	1600	0.71	
Group II Purina + 7 vitamins _____	34	45	290	74	10	0.19	4800	0.71	600
Group III Purina + 7 vitamins + yeast extract _____	34	45	290	80	10	0.50	4800	0.81	600
Group IV Purina + spleen extract _____	3.3	6.6	43	14	1.9	0.22	1700	1.4	
Group V Purina + 3 vitamins _____	3.7	45	290	74	2.1	0.19	1600	0.71	
Group VI Rice-Carrot _____	3.4	0.22	17	7.1	0.26	0.016	140	0.05	
Group VII Purina + raw egg white _____	3.3	7.2	32	13	1.7	0.18	1400	0.63	

*Calculated on basis of material with "potency" 40,000.

acid originally present in the Purina. Group VI was fed boiled whole rice supplemented with salts and casein to the extent where these ingredients were present to an adequate degree, and in addition each mouse received a slice of raw carrot each day. Group VII was maintained on Purina plus 20% egg white powder.

The experimental diets were carefully homogenized by use of a ball mill.

Tumor Transplantation

For tumor transplantation, a mammary carcinoma spontaneous in origin and which had become stabilized by numerous generations of transplants was selected. Mice with tumors of about 2 grams in weight were anesthetized and bled by decapitation. The tumors were aseptically removed and only those used which were free from necrosis and hemorrhagic areas. The cancer tissue thus obtained was made into a suspension by forcing it through a muslin cloth. 10 ml. of saline solution (0.8% NaCl) was added for each ml. of tumor tissue. Each animal received 0.2 ml. of the suspension or 0.02 ml. of tumor tissue subdermally in the dorsal area just posterior to the cervical vertebrae, by hypodermatic injection, using a number 18 needle. Every effort was made to keep this operation aseptic throughout.

Hemoglobin Determination

The hemoglobin concentration of the blood was measured just prior to the initiation of the experiment and at weekly intervals thereafter. Blood was taken from the tail for these readings and the hemoglobin level determined by use of the Evelyn Colorimeter according to the method described by Evelyn (2).

Tumor Measurement

The mice were observed daily and records kept of the time of appearance of each tumor. Tumor growth was recorded by measuring two ways across the tumor and using the product of these two diameters as an index of size. A weight record of each mouse was also obtained and a record kept of death dates.

RESULTS

The data are summarized in Figures 1, 2, 3, 4, and Tables I and II. It will be noted that there was no striking deviation with respect to tumor growth, or time of death of the implanted animals by any one group as compared to the others. However, some apparently valid differences with respect to tumor susceptibility are evident.

TABLE II

Effect of Diet on Hemoglobin Concentration in Tumor-Bearing Mice

	1st Week on Diet Hemoglobin Grams Per Cent	1st Week After Implant Hemoglobin Grams Per Cent	2d Week After Implant Hemoglobin Grams Per Cent	3d Week After Implant Hemoglobin Grams Per Cent
Group I Purina (Control) _____	16.99	14.74	10.40	5.83
Group II Purina + 7 vitamins_____	16.46	14.29	13.36	6.64
Group III Purina + 7 vitamins + yeast extract_____	17.16	14.74	13.58	6.67
Group IV Purina + spleen extract	17.95	15.67	13.04	6.09
Group V Purina + 3 vitamins_____	17.84	15.70	13.14	7.22
Group VI Rice-carrot _____	15.91	15.28	12.83	7.98
Group VII Purina + egg white_____	16.71	15.15	13.03	6.70

In group II (Purina plus 7 vitamins), group V (Purina plus 3 vitamins) and group VI (rice-carrot) there occurred 3 non-"takes" of the cancer implant in each. There were 2 non-"takes" in group III (Purina plus 7 vitamins plus yeast extract). All the other groups—group I (Purina), group IV (Purina plus spleen extract, and group VII (Purina plus egg white powder) gave 100% "takes."

Table II records the effect of the various procedures on the hemoglobin concentration. In the period when the mice were on the diets but before

implantation, group IV (Purina plus spleen extract) and group V (Purina plus 8 vitamins) were associated with a distinct rise in hemoglobin level, while the rice-casein-carrot diet occasioned a lower level in this respect. After the implants were made the hemoglobin of all the groups underwent a steady decline in concentration in association with the growth of the tumors. This reaction of the hemoglobin level to tumor growth has been considered in detail in a previous publication (3).

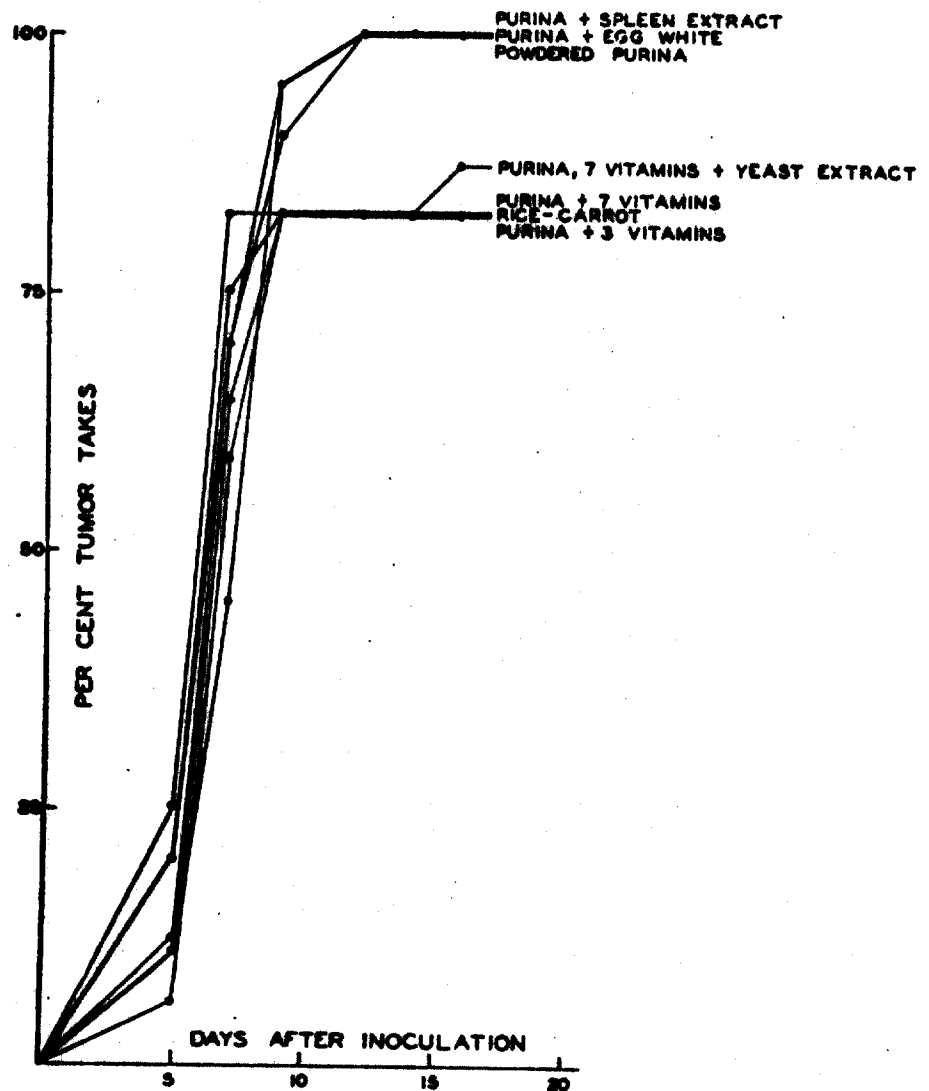


Fig. 1. Effect of Diet on Infection from Tumor Transplants among Mice.

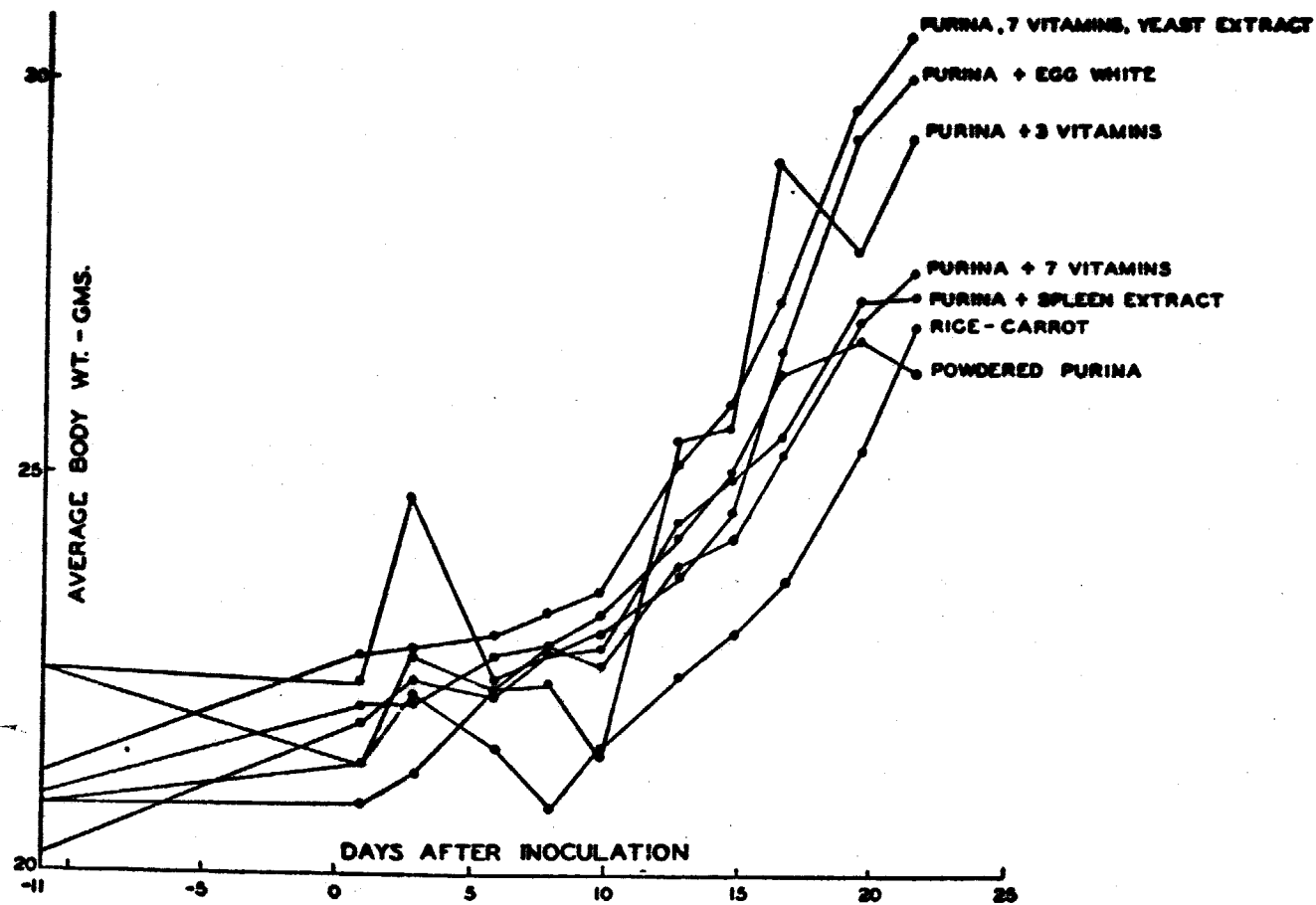


Fig. 2. Effect of Diet on Growth of Mice Bearing Transplanted Tumors.

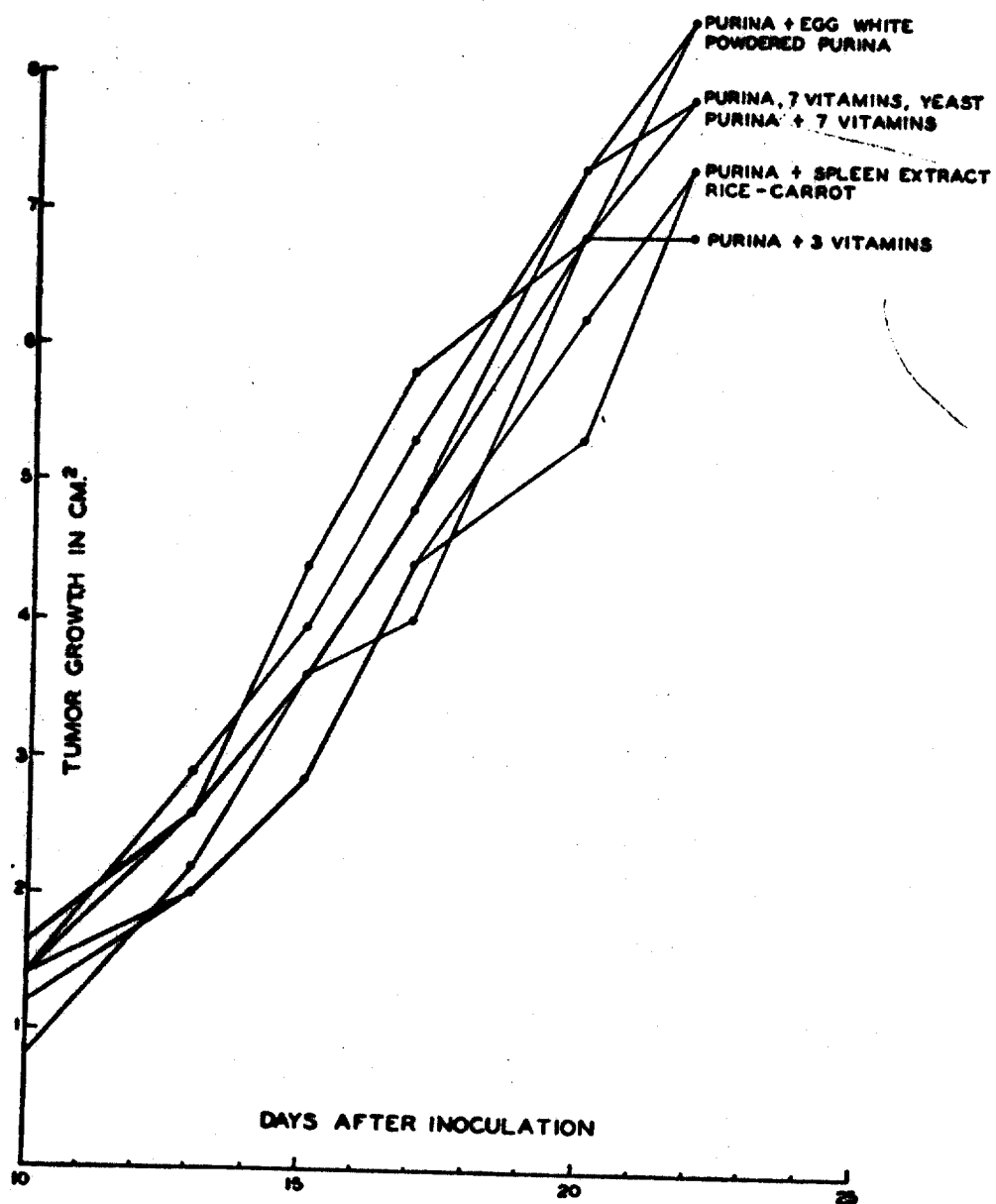


Fig. 3. Effect of Diet on Growth of Tumor Transplants in Mice.

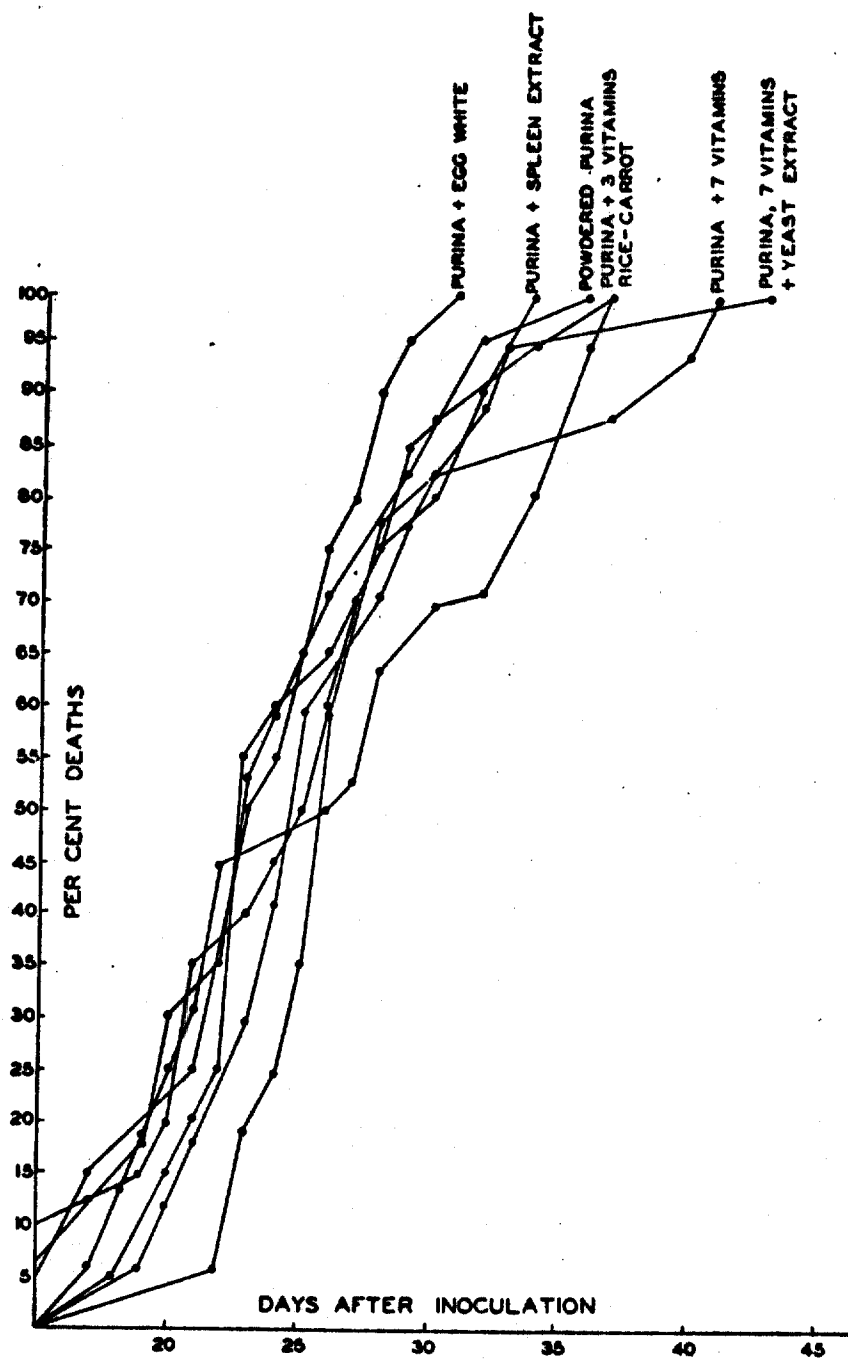


Fig. 4. Effect of Diet on Deaths from Transplanted Tumors.

DISCUSSION

It is evident that a small but definite effect on cancer "takes" was produced in the three groups receiving a diet containing several times the normal requirements of some of the B vitamins. Of these sixty mice about 18 per cent were resistant to the growth of the implanted tumor tissue. This is in contrast to the 100 per cent susceptibility shown by the 60 mice of the three groups which were maintained on diets, the vitamin content of which was approximately the same as that of Purina.

The group of 20 mice which were maintained on the rice-carrot diet also manifested resistance to the extent of 15 per cent non-"takes" to the tumor implants. The diet in this instance, as is shown by Table I, was low in most of the B vitamins, but other factors may have been responsible for the lowered tumor susceptibility.

Tannenbaum (4) has shown in a recent paper that any diet which tends to keep an animal below normal in weight will also be inimical to tumor formation and growth. In the present study this factor was not concerned, since as the data show, the animals of the various groups were approximately the same in body and tumor weight.

It is to be noted that the three groups in which no non-"takes" occurred were the ones fed diets with practically the same vitamin content. Groups IV and VII were included for other reasons. Group IV (Purina plus spleen extract) was used because of the results Lewisohn and coworkers (5) reported for this diet. There was the possibility that some vitamin which is yet unknown might be present in effective quantities in this material. We were unable to confirm Lewisohn's results. Group VII (Purina plus egg white) was used in the expectation of obtaining a biotin deficiency by this means. We have since learned that for the period of the experiment little if any biotin deficiency should be expected.

SUMMARY

A study was made of the effect of diets of different vitamin levels on the "takes" and growth of tumor transplants. 140 dba mice divided into 7 groups of 20 each were utilized.

It was found that the 3 groups which were maintained on diets in which the level of several of the B vitamins was several times greater than on Purina dog chow, 10 to 15 per cent of the mice were resistant to the growth of cancer implants as compared with complete susceptibility for the controls.

One group maintained on a low B vitamin rice-carrot diet gave 20 per cent non-"takes." In this instance, other factors may have been responsible for the changed susceptibility.

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Report

By: Dr. E. Thiess
Section: VI/Med.
Date: February 1, 1963
Subject: Topic 2090
Es M/8212
M/8298

Ro 1-9296 = thiamine thiocyanate
Ro 1-9296/1 = thiamine dithiocyanate
Ro 1-3322 = thiamine hydrochloride

Toxicity Testing by Oral Administration to Rats for 6 Weeks

SUMMARY

Ro 1-9296 (thiamine thiocyanate) and Ro 1-9296/1 (thiamine dithiocyanate) had been considered as substitutes for the physically less stable Ro 1-3322 (vitamin B₁ hydrochloride). In a 6-week study on rats, the three substances were compared at doses of 500 and 1500 mg/kg.

Only the hydrochloride was well tolerated. The dithiocyanate at the higher dosage is strongly toxic, while the monothiocyanate at both dosage levels showed signs of a slight, not entirely unequivocal toxicity (see discussion).

Because of their greater physical stability, vitamin B₁-mono or dithiocyanate have been considered by Pharma PT (I. R. No. 179 of Pharma PT or VI/VEF of 9/22/61) as substitutes for Vitamin B₁-hydrochloride. The present report deals with a comparative, sub-acute toxicity testing.

EXPERIMENTAL PROCEDURE

Groups containing 4 male and 4 female white rats of the laboratory's own strain weighing 80-90 g received the test substances 5 days per week at doses of 500 and 1500 mg/kg by stomach tube. A control group received a like amount of water by stomach tube. A normal diet was fed. The experiment lasted 6 weeks.

STUDIES CARRIED OUT

1. Body Weight and General Health

were controlled on all work days. In the second and fifth weeks, feed

measurements were conducted.

2. Blood Studies

At the conclusion of the experiment, hemoglobin content, hematocrit value, reticulo-, thrombo- and leukocyte count were determined for all animals and a differential white blood count made.

3. Urine Analysis

At the conclusion of the experiment, the urine sediments of the highest dosed animals were studied microscopically.

4. Liver Function and Enzyme Tests

At the end of the third week and at the close of the experiment, the following tests were carried out, likewise on the highest dosed animals:

- a) Serum Glutamate - Oxalacetate Transaminase (SGOT),
- b) Serum Glutamate - Pyruvate Transaminase (SGPT),
- c) Lactic Acid - Dehydrogenase (LDH),
- d) Sorbite - Dehydrogenase (SDH),
- e) Thymol Turbidity Test

In addition, at the conclusion of the experiment, the ability of the liver to separate out bromine sulphthaline was tested.

5. Histological Studies

Heart, liver, kidney and testicles were examined microscopically.

RESULTS

1. Body Weight and General Health (see Fig. 1 for body weight curve):

Ro 1-9296 was tolerated in both dosage groups without deleterious effects on the general health. The animals receiving 500 mg/kg gained weight well, the animals receiving 1500 mg/kg remained slightly behind the controls with regard to weight.

One of the rats given 500 mg/kg died in the fifth week of the experiment after inadvertent insertion of the tube into the lungs. Three of the rats from the higher dose group died during the course of the experiment as a result of inadvertent insertion of the tube into the lungs.

Ro 1-9296/1: The animals given 1500 mg/kg lost weight sharply after the first insertion of the tube. After the second application, one rat died and the others were in such poor general condition that this group was prematurely terminated. With the rats receiving 500 mg/kg, it became apparent during the course of the experiment that, erroneously,

seven females and only one male had been included in this group. The weight increase of the females could not be distinguished from that of the controls. The male gained even more weight than the controls but died at the end of the fourth week of the experiment because of an inadvertent insertion of the tube into the lungs. For the same reason, a female died at the end of the third week. The general condition of the remaining rats of this group during the entire duration of the experiment is good.

Ro 1-3322: The preparation was tolerated in both dosage groups without deleterious effects on the general health. For males and females, 1500 mg/kg caused a slight depression of growth. For the females, the weight increase was even more reduced by 500 mg/kg than, paradoxically, by the higher dose. Of the animals given 500 mg/kg, one female died; of the animals given 1500 mg/kg, one female and one male died due to inadvertent insertion of the tube into the lungs.

2. Blood Studies

Hemoglobin content, hematocrits, reticulo-, thrombo-, and leukocyte counts as well as the differential white blood counts of the animals given the three preparations showed no differences from the controls (see Table 1 for average values).

3. Urine Analysis

No pathological changes were found in the sediment.

4. Liver Function and Enzyme Tests

With the animals given the three preparations, the determination of SGOT, SGPT, LDH, SDH and the thymol turbidity test gave no indication of liver damage, neither at the end of the third week nor at the conclusion of the experiment. A BSP-test taken at the end of the experiment was also negative.

5. Histological Studies

Ro 1-9296: In three of the males given 1500 mg/kg, an incomplete inhibition of development of the germinal epithelium in the testes was observed. In many tubules, only spermatogonia and Sertoli-cells were found, in others primary and secondary spermatocytes are also seen and, occasionally, also spermatids and isolated spermatozoa. The inhibition of development is completely different in different fields of view and not confined to a definite level. Two of these rats exhibited poor general condition before death. Whether this could explain the changes in the testicles is questionable, although possible. In any case, an effect of the preparation cannot be excluded.

No other significant changes of the organs were observed in the two dose groups.

Ro 1-9296/1: With the males given 1500 mg/kg, the testicles are still immature since the animals died by the second day of the experiment. An effect directly conditional on the preparation is not likely. On the contrary, a strong, coarse vacuolization of the epithelium of the distal nephron (collecting tubules) was found in two animals of this group. Besides, the neighboring kidney walls are clearly lipoid-poor. Liver and heart are histologically of no special significance.

With the animals given 500 mg/kg, the testicles of the sole male of this group were completely normal. Disregarding occasional slight fluctuations in the size of the nucleus of liver cells, there were no preparation-related pathological organ changes in this group.

Ro 1-3322: Neither with the animals given 1500 mg/kg nor with those given 500 mg/kg were there any preparation-related organ changes.

DISCUSSION

The results of this experiment are not completely unequivocal. The only preparation which -- ignoring a slight reduction of growth of the animals concerned -- was tolerated unhesitatingly and without any sort of indication of a toxic side effect is Ro 1-3322.

In three of the rats given 1500 mg/kg of Ro 1-9296, an incomplete inhibition of development of the testicular germinal epithelium was found. It is not clear, however, to what degree this change is due to the preparation directly or to a reduced general condition.

Ro 1-9296/1 was tolerated very poorly in doses of 1500 mg/kg. The animals lost weight sharply even after the first application and were in such a miserable general condition that the group had to be terminated prematurely. In the histological study, a strong hydropic epithelium degeneration of the distal nephron in the kidneys was seen in two, and a clear lipoid deficiency of the adjacent kidney wall is seen in the majority of these animals. With the animals given 500 mg/kg, signs of discreet liver parenchyma damage were occasionally observed.

On the basis of the findings discussed above, a clinical use of Ro 1-9296/1 (thiamine dithiocyanate) is to be prohibited; a clinical test of Ro 1-9296 (thiamine thiocyanate) can be approved only with hesitation.

It is interesting that the acute toxicity testing (RCR Dr. Pellmont No. 60054 of 1/16/1962) by oral administration to mice yielded a similar gradation of compatibility as reported in the present article.

<u>Preparation</u>	<u>LD 50% Mouse</u>
Ro 1-9296	2500 mg/kg p.o.
Ro 1-9296/1	1800 mg/kg p.o.
Ro 1-3322	> 5000 mg/kg p.o.

In this summary, reference should be made once more to the good acute compatibility of No 1-4788 (thiamine monophosphate) found by Dr. Pellmont.

Hematological Studies with Groups of Rats given Ro 1-3322, Ro 1-9296, and Ro 1-9296/1

Tabelle 1 : Haematologische Untersuchungen mit Ro 1-3322-, Ro 1-9296- und Ro 1-9296/1 - behandelten Rattengruppen

Studies durchgeföhrte carried out Untersuchungen	Ro 1-9296				Ro 1-9296/1		Ro 1-3322				Kontrolle (c)	
	500 mg/kg p.o. männl. ^(a) weibl. ^(b)		1500 mg/kg p.o. männl. weibl.		500 mg/kg p.o. männl. weibl.		500 mg/kg p.o. männl. weibl.		1500 mg/kg p.o. männl. weibl.		männl.	weibl.
(d) durchschnittl. Körpergewichts- zunahme in g in % d. Kontr.	124,0 108,2	70,0 87,2	91,8 80,2	65,0 81,0	91,5 79,9	79,5 99,1	113,0 98,7	58,0 72,3	117,4 102,6	67,8 84,5	114,5 100,0	80,3 100,0
Haemoglobin	14,2	14,4	14,8	14,1	14,1	14,2	14,4	14,8	13,2	14,8	13,7	13,8
Haematocrit	43,3	43,5	45,0	41,5	42,3	42,3	43,2	44,0	40,0	45,7	43,2	41,2
Leuko. x1000	8,0	9,0	9,0	7,8	10,6	9,9	9,6	10,4	11,8	12,1	11,1	9,1
Stab.	-	0,5	-	-	0,3	-	0,5	0,3	-	0,3	-	0,25
Segment.	19,0	17,0	28,0	27,2	18,7	11,3	22,0	15,3	22,7	25,3	24,5	18,75
Lympho	75,3	78,5	71,0	74,0	77,3	87,7	76,3	82,6	76,7	70,7	72,5	77,25
Mono.	1,0	0,75	1,0	0,5	0,3	-	-	0,3	-	-	0,25	1,0
Eosino.	4,7	4,3	-	0,5	3,3	1,3	1,25	1,0	0,6	3,6	2,75	2,75
Baso.	-	-	-	0,25	-	0,3	-	0,3	-	-	-	-
Reticulo.in %	16,0	10,0	6,0	4,7	15,3	18,7	24,0	11,3	54,0	12,7	26,0	20,0
Thrombo.x1000	496,3	487,0	436,0	421,0	454,7	481,7	470,0	433,3	581,3	582,7	450,5	535,0

Note: In numeric entries, read a comma as a decimal point, e.g., "124,0" = 124.0

a) Male

b) Female

c) Control

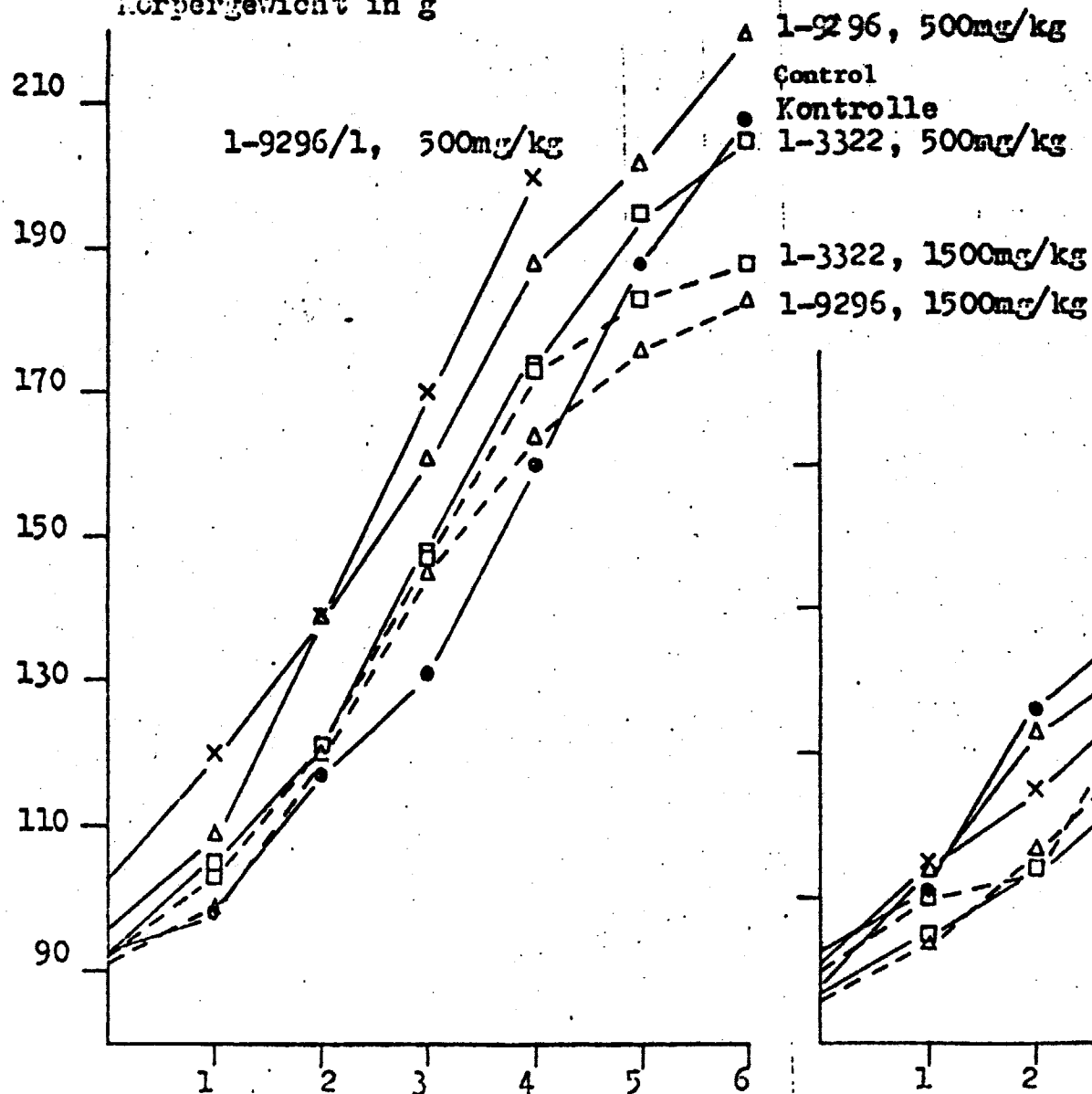
d) Average Body Weight taken in g, in % of control

Abb. 1

Weight Curve
Gewichtskurve Ro 1-9296, Ro 1-9296/1, Ro 1-3322

Male Rats per os
männliche Ratten per os

Body Weight in g
Körpergewicht in g



Female Rats per os
weibliche Ratten per os

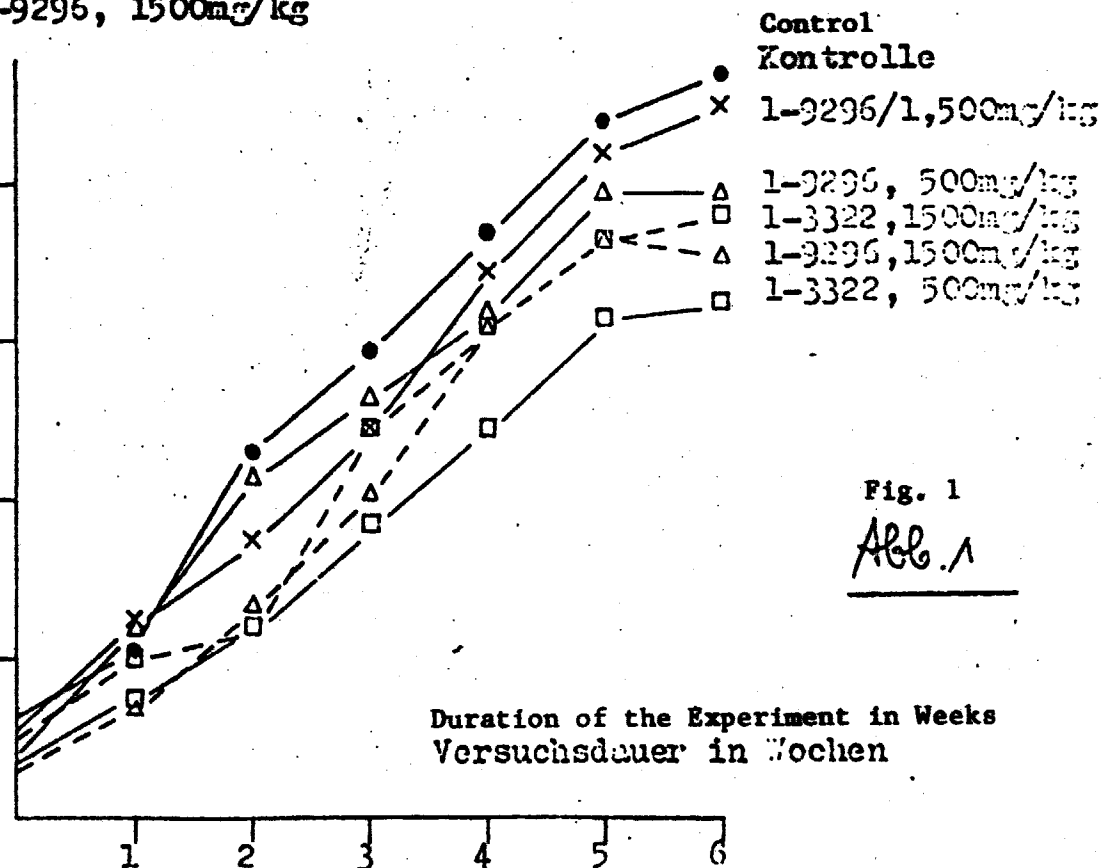


Fig. 1

Abb. 1

Duration of the Experiment in Weeks
Versuchsdauer in Wochen

Thiess-'63
Bib No. 4004

Dr. Thiess

Report

February 1, 1963

Toxicity Testing of Thiamine Thiocyanate, Thiamine Dithiocyanate and Thiamine Hydrochloride by Oral Administration to Rats for 6 weeks.

Translation of Author's Summary

Ro 1-9296 (thiamine thiocyanate) and Ro 1-9296/1 (thiamine dithiocyanate) had been considered as substitutes for the physically less stable Ro 1-3322 (vitamin B₁ hydrochloride). In a 6-week study on rats, the three substances were compared at doses of 500 and 1500 mg/kg.

Only the hydrochloride was well tolerated. The dithiocyanate at the higher dosage is strongly toxic, while the monothiocyanate at both dosage levels showed signs of a slight, not entirely unequivocal toxicity.

Abstract

Groups containing 4 male and 4 female white rats of the laboratory's own strain weighing 80-90 g received the test substances 5 days per week at doses of 500 and 1500 mg/kg by stomach tube. A control group received a like amount of water by stomach tube. A normal diet was fed. The experiment lasted 6 weeks.

Thiamine hydrochloride was tolerated at both dosages without deleterious effect on general condition. 1500 mg/kg caused a slight depression of growth in both males and females. In the females, growth was depressed even more by 500 mg/kg than by the higher dose. Of the group which received 500 mg/kg one female died, and in the 1500 mg/kg group one male and one female died due to the tube getting into the lungs by mistake.

Hemoglobins, hematocrits, blood counts and differential white counts showed no differences from the controls (Table 1). Urinalyses showed no pathological changes. Liver function and enzyme tests (SGOT, SGPT, LDH, SHH, thymol turbidity) gave no signs of liver damage at three weeks and at the end of the experiment. A BSP test was also negative at the end of the experiment. No histological changes were found in hearts, livers, kidneys, and testes.

Translated by WRSullivan/jk
12/13/71

ROTECKENRAPPORT

von : Dr. E. Theiss

Abteilung : VI/Med.

Datum : 1. Februar 1963

Betrifft : Thema 2090

ES M/6212

M/6298

Ro 1-9296 = Aneurin-rhodanid

Ro 1-9296/1 = Aneurin-dirhodanid

Ro 1-3322 = Aneurin-hydrochlorid

Toxizitätsprüfung bei oraler Verabreichung an Ratten während 6 Wochen

Zusammenfassung:

Ro 1-9296 und Ro 1-9296/1 waren als Ersatz für das physikalisch instabilere Ro 1-3322 (Vitamin B₁-hydrochlorid) in Erwägung gezogen worden.

In einem 6-wöchigen Versuch an Ratten wurden die 3 Präparate in Dosen von 500 und 1500 mg/kg vergleichend geprüft.

Ro 1-3322 allein wurde anstandslos vertragen. Ro 1-9296/1 ist in der hohen Dosierung stark toxisch, während Ro 1-9296 in beiden Dosierungen Zeichen einer geringen, nicht ganz eindeutigen Toxizität aufwies (s. Diskussion Seite 5/6).

Tss/kpf

Theiss

Wegen ihrer grösseren physikalischen Stabilität wurde von Pharma PT (s. I.M. Nr. 179 von Pharma PT an VI/VEF vom 22.9.1961) der Ersatz von Vitamin B₁-hydrochlorid durch Vitamin B₁-mono- bzw. dirhodanid in Erwägung gezogen. Der vorliegende Rapport berichtet über eine vergleichende subakute Toxizitätsprüfung.

VERSUCHSANORDNUNG

Gruppen von je 4 männlichen und 4 weiblichen Albinoratten eigener Zucht mit einem Anfangsgewicht von 80 - 90 g erhielten an den 5 Arbeitstagen der Woche täglich 500 bzw. 1500 mg/kg der zu testenden Substanzen oral per Schlundsonde verabreicht. Eine gleichartige Kontrollgruppe erhielt analoge Mengen H₂O per Schlundsonde. Die Diät bestand aus Normalkost. Die Versuchsdauer betrug 6 Wochen.

DURCHFÜHRTE UNTERSUCHUNGEN

1. Körpergewicht und Allgemeinbefinden

wurden an allen Arbeitstagen kontrolliert. In der 2. und 5. Woche wurden Futtermessungen durchgeführt.

2. Blutuntersuchungen:

Bei Versuchsabschluss wurden von allen Tieren Haemoglobingehalt, Haematokritwert, Reticulo-, Thrombo- und Leukozytenzahl bestimmt und ein weisses Differentialblutbild angefertigt.

3. Urinanalyse:

Von den höchstdosierten Tieren wurde bei Versuchsabschluss das Urinsediment mikroskopisch untersucht.

4. Leber-Funktion und Enzymteste:

Am Ende der 3. Versuchswoche und bei Versuchsabschluss wurden folgende Tests jeweils bei den höchstdosierten Tieren durchgeführt:

- a) Serum Glutamat-Oxalacetat Transaminase (SGOT),
- b) Serum Glutamat-Pyruvat Transaminase (SGPT),
- c) Milchsäure-Dehydrogenase (LDH),
- d) Sorbit-Dehydrogenase (SDH)
- e) Thymoltrübungstest

Bei Versuchsabschluss wurde ausserdem die Fähigkeit der Leber, Bromsulphthalein (BSP) auszuscheiden, getestet.

5. Histologische Untersuchungen:

Ners, Leber, Niere, Hoden wurden mikroskopisch untersucht.

ERGEBNISSE

1. Körpergewicht und Allgemeinbefinden (Körpergewichtskurve s. Abb.1):

No 1-9296 wird in beiden Dosiagruppen ohne Beeinträchtigung des Allgemeinbefindens vertragen. Die mit 500 mg/kg behandelten Tiere nehmen gut an Körpergewicht zu, die mit 1500 mg/kg behandelten Tiere bleiben gewichtsmässig leicht hinter den Kontrollen zurück.

Eine mit 500 mg/kg behandelte Ratte stirbt in der 5. Versuchswoche nach versehentlicher Sondierung der Lungen.

Drei Ratten aus der oberen Dosiagruppe sterben während des Versuchsverlaufs infolge versehentlicher Lungensondierung.

No 1-9296/1: Die mit 1500 mg/kg behandelten Tiere nehmen schon nach der ersten Sondierung stark an Gewicht ab. Nach der 2. Applikation stirbt eine Ratten, die anderen befinden sich in einem so schlechten Allgemeinzustand, dass diese Gruppe vorzeitig abgeschlossen wird. Bei den mit 500 mg/kg behandelten Ratten stellt sich während des Versuchsverlaufs heraus, dass irrtümlich 7 Weibchen und nur 1 Männchen in dieser Gruppe eingesetzt wurde. Die Gewichtszunahme der Weibchen unterscheidet sich nicht von den Kontrollen. Das Männchen nimmt sogar stärker an Gewicht zu als die Kontrollen, geht aber am Ende der 4. Versuchswoche an einer versehentlichen Lungensondierung ein. Aus dem gleichen Grund stirbt ein Weibchen am Ende der 3. Woche. Der Allgemeinzustand der restlichen Ratten dieser Gruppe ist während der ganzen Versuchsdauer gut.

No 1-3322: Das Präparat wird in beiden Dosierungsgruppen ohne Beeinträchtigung des Allgemeinbefindens vertragen. Bei Männchen und Weibchen bewirken 1500 mg/kg eine leicht verzögerte Gewichtsentwicklung. Bei den Weibchen wird die Gewichtszunahme paradoxerweise durch 500 mg/kg noch stärker reduziert als durch die höhere Dosis. Von den mit 500 mg/kg behandelten Tieren stirbt ein Weibchen, von den mit

1500 mg/kg behandelten Tieren je ein Weibchen und ein Männchen an versehentlicher Sondierung der Lungen.

Die durchschnittliche Futteraufnahme wird durch keines der Präparate beeinflusst.

2. Blutuntersuchungen:

Hämoglobingehalt, Hämatokritwert, Reticulo-, Thrombo- und Leukozytenzahl, sowie das weisse Differentialblutbild der mit den 3 Präparaten behandelten Tiere unterscheiden sich in keiner Weise von den Kontrollen (Durchschnittswerte s. Tab. 1).

3. Urinanalysen:

Im Sediment wurden keine pathologischen Veränderungen gefunden.

4. Leber-Funktion und Enzymteste:

Bei den mit den 3 Präparaten behandelten Tieren ergaben die Bestimmung der

SGOT

SGPT

LDH

SDH und des

Thymoltrübungstests

weder in der 3. Woche noch bei Versuchsabschluss einen Hinweis auf eine Leberschädigung. Ebenso war der bei Versuchsabschluss vorgenommene BSP-Test negativ.

5. Histologische Untersuchungen:

No 1-9296: Bei 3 der mit 1500 mg/kg behandelten Männchen beobachtet man in den Hoden einen unvollständigen Reifungsstop des Keimepithels. In vielen Tubuli finden sich nur Spermatogonien und Sertoli-Zellen, in anderen sieht man zusätzlich Spermatozyten I. und II. Ordnung, zuweilen auch Spermatiden und vereinzelt Spermatozoen. Die Reifungshemmung ist in verschiedenen Gesichtsfeldern ganz unterschiedlich und nicht auf eine bestimmte Ebene beschränkt. Zwei dieser Ratten wiesen vor dem Tod einen schlechten Allgemeinzustand auf. Ob damit die Hodenveränderungen erklärt werden können, ist fraglich, wenn auch möglich. Jedenfalls kann ein Präparat-Effekt nicht ausgeschlossen werden.

Andere signifikante Organveränderungen wurden in keiner der beiden Dosisgruppen beobachtet.

Ro 1-9296/1: Bei den mit 1500 mg/kg behandelten Männchen sind die Hoden noch unreif, da die Tiere bereits am 2. Versuchstag getötet wurden. Ein direkter Präparat-bedingter Effekt ist nicht wahrscheinlich. Dagegen findet sich bei 2 Tieren dieser Gruppe eine starke grobe Vakuolisierung der Epithelien des distalen Nephrons (Sammelkapillchen). Ausserdem sind die Nebennierenrinden deutlich Lipoid-arm. Leber und Herz sind histologisch unauffällig.

Bei den mit 500 mg/kg behandelten Tieren war der Hoden des einzigen Männchens dieser Gruppe völlig normal. Abgesehen von gelegentlichen, leichten Kerngrössenschwankungen in Leberzellen fanden sich keine Präparat-bedingten pathologischen Organveränderungen in dieser Gruppe.

Ro 1-3322: Weder bei den mit 1500 mg/kg, noch bei den mit 500 mg/kg behandelten Tieren findet man Präparat-bedingte Organveränderungen.

DISKUSSION

Die Ergebnisse dieser Versuchs sind nicht ganz eindeutig. Das einzige Präparat, welches - abgesehen von einer leicht reduzierten Gewichtsentwicklung der behandelten Tiere - anstandslos und ohne irgendwelche Zeichen eines toxischen Nebeneffektes vertragen wurde, ist Ro 1-3322.

Bei drei der mit 1500 mg/kg Ro 1-9296 behandelten Ratten findet man eine unvollständige Reifungshemmung des testikulären Keimepithels, wobei aber nicht klar ist, inwieweit diese Veränderung auf das Präparat direkt oder aber auf einen reduzierten Allgemeinzustand zurückzuführen ist.

Ro 1-9296/1 wird in Dosen von 1500 mg/kg sehr schlecht vertragen. Die Tiere nehmen schon nach den ersten Applikationen stark an Gewicht ab und befinden sich in so miserablen Allgemeinzustand, dass die Gruppe vorzeitig abgeschlossen werden muss. Bei der histologischen Untersuchung sieht man eine starke hydropische Epitheldegeneration des distalen Nephrons in den Nieren von zwei und eine deutliche Lipoidverarmung der Nebennierenrinden in der Mehrzahl dieser Tiere. Bei den mit 500 mg/kg behandelten Tieren beobachtet man gelegentlich Anzeichen einer diskreten Leberparenchymschädigung.

Auf Grund der oben diskutierten Befunde verbietet sich eine klinische Verwendung von Ro 1-9296/1 (Thiamin-dirhodanid); einer klinischen Prüfung von Ro 1-9296 (Thiamin-monorhodanid) kann nur mit Bedenken zugestimmt werden.

Es ist interessant, dass die akute Toxizitätsprüfung (RCR Dr. Pellmont No. 60054 vom 16.1.1962) bei oraler Verabreichung an Mäusen eine ähnliche Abstufung der Verträglichkeit ergeben hat wie die in dem vorliegenden Rapport berichtete.

<u>Präparat</u>	<u>DL 50 % Maus</u>
Ro 1-9296	2500 mg/kg p.o.
Ro 1-9296/1	1800 mg/kg p.o.
Ro 1-3322	>5000 mg/kg p.o.

In diesen Zusammenhang sei nochmals auf die von Dr. Pellmont gefundene gute akute Verträglichkeit von Ro 1-4788 (Aneurin-monophosphat) hingewiesen.

Tee/kpf

<u>HIST.-NO.</u>	120 627 - 120 631
	120 670 - 120 705
	120 744 - 120 747
	121 059 - 121 063
	121 139 - 121 148
	121 155 - 121 163
	121 170 - 121 174
	121 504 - 121 508
	121 627 - 121 795

Tabelle 1 : Haematologische Untersuchungen mit Ro 1-3322-, Ro 1-9296- und Ro 1-9296/1 - behandelten Rattengruppen

durchgeführte Untersuchungen	Ro 1-9296				Ro 1-9296/1		Ro 1-3322				Kontrolle	
	500 mg/kg p.o.		1500 mg/kg p.o.		500 mg/kg p.o.		500 mg/kg p.o.		1500 mg/kg p.o.		männl.	weibl.
	männl.	weibl.	männl.	weibl.	männl.	weibl.	männl.	weibl.	männl.	weibl.	männl.	weibl.
durchschnittl. Körpergewichtszunahme in g in % d. Kontr.	124,0 108,2	70,0 87,2	91,8 80,2	65,0 81,0	91,5 79,9	79,5 99,1	113,0 98,7	58,0 72,3	117,4 102,6	67,8 84,5	114,5 100,0	80,3 100,0
Haemoglobin	14,2	14,4	14,8	14,1	14,1	14,2	14,4	14,8	13,2	14,8	13,7	13,8
Haematocrit	43,3	43,5	45,0	41,5	42,3	42,3	43,2	44,0	40,0	45,7	43,2	41,2
Leuko. x1000	8,0	9,0	9,0	7,8	10,6	9,9	9,6	10,4	11,8	12,1	11,1	9,1
Stab.	-	0,5	-	-	0,3	-	0,5	0,3	-	0,3	-	0,25
Segment.	19,0	17,0	28,0	27,2	18,7	11,3	22,0	15,3	22,7	25,3	24,5	18,75
Lympho	75,3	78,5	71,0	74,0	77,3	87,7	76,3	82,6	76,7	70,7	72,5	77,25
Mono.	1,0	0,75	1,0	0,5	0,3	-	-	0,3	-	-	0,25	1,0
Eosino.	4,7	4,3	-	0,5	3,3	1,3	1,25	1,0	0,6	3,6	2,75	2,75
Baso.	-	-	-	0,25	-	0,3	-	0,3	-	-	-	-
Reticulo.in %	16,0	10,0	6,0	4,7	15,3	18,7	24,0	11,3	54,0	12,7	26,0	20,0
Thrombo.x1000	496,3	487,0	436,0	421,0	454,7	481,7	470,0	433,3	581,3	582,7	450,5	535,0

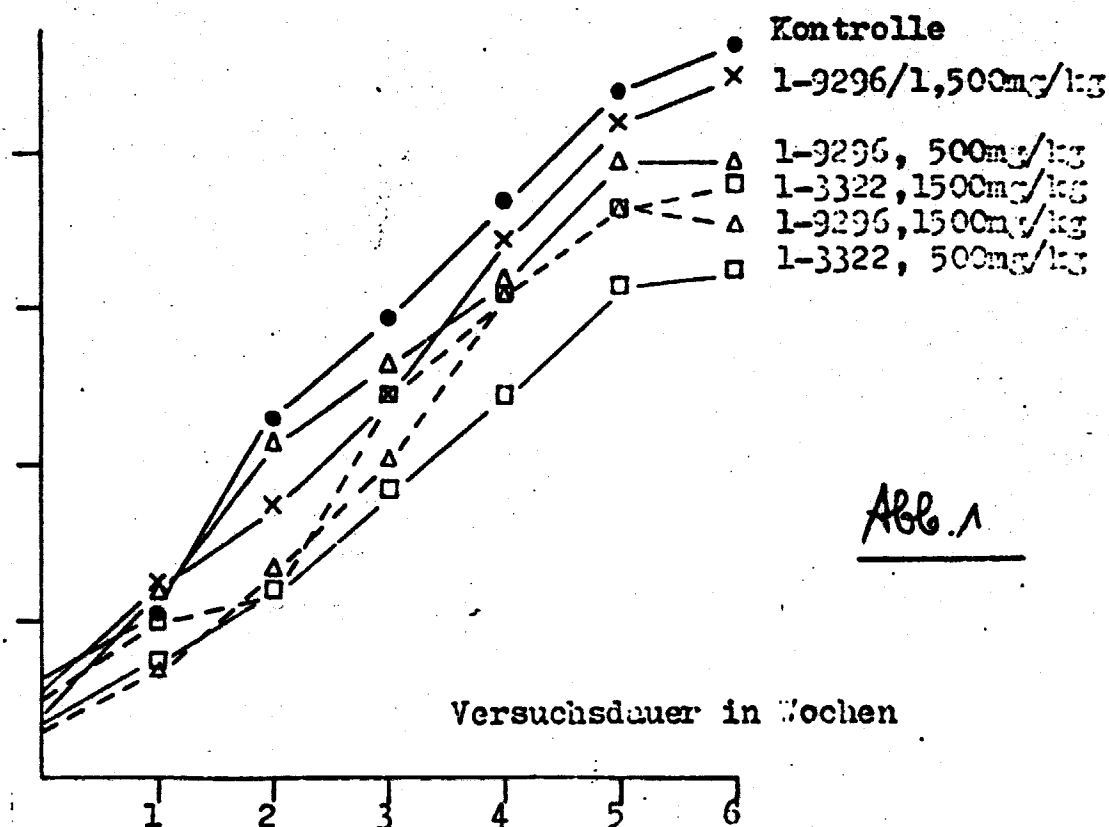
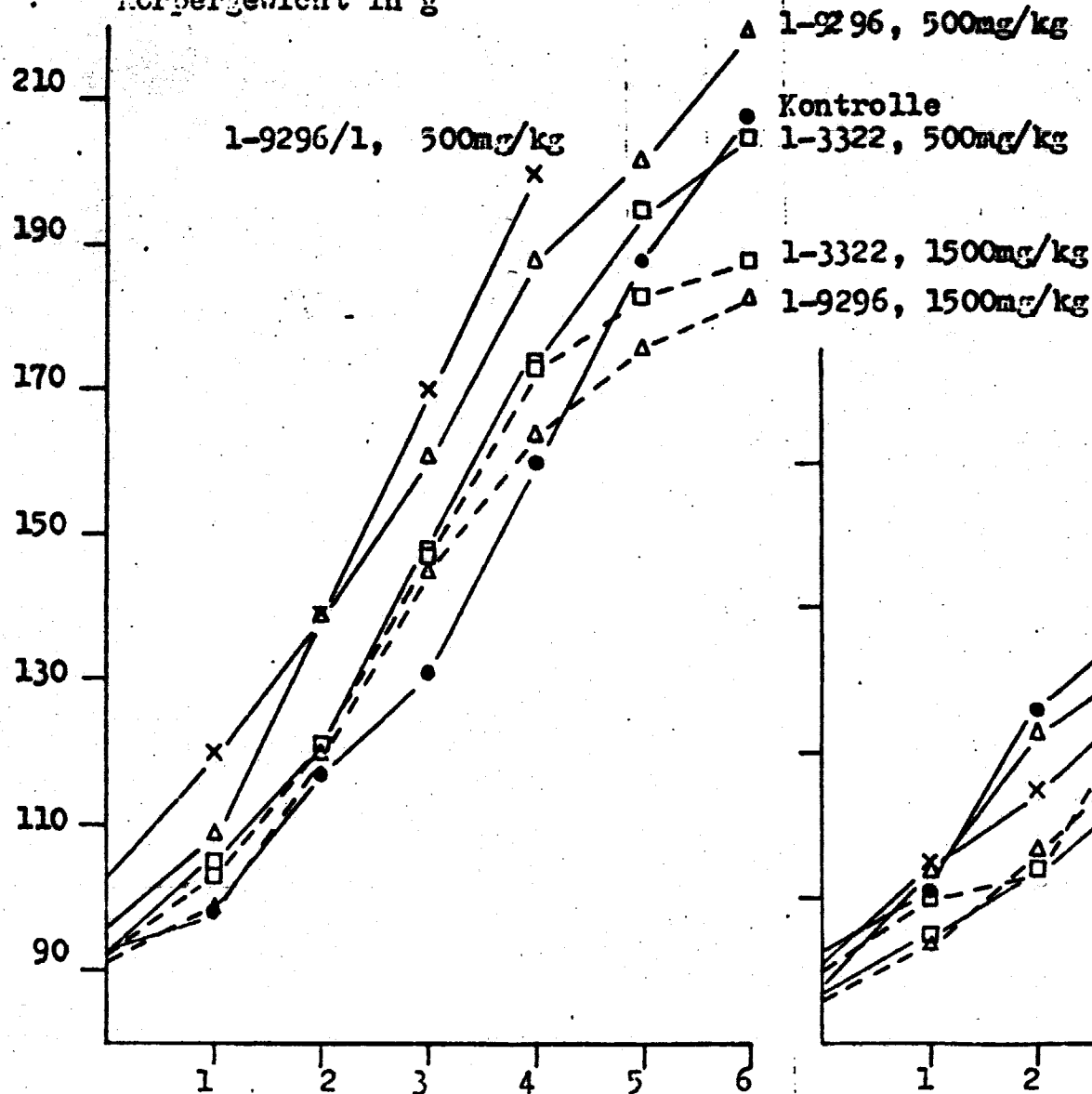
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Gewichtskurve Ro 1-9296, Ro 1-9296/1, Ro 1-3322

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Körpergewicht in g



Versuchsdauer in Wochen

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THIAMINE HYDROCHLORIDE

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OBSERVATIONS ON THE MECHANISM OF THIAMINE HYDROCHLORIDE ABSORPTION IN MAN

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(Received 12 January 1972)

SUMMARY

1. The intestinal absorption of [^{35}S]thiamine hydrochloride was investigated in healthy subjects, malnourished alcoholics and a patient with resection of the jejunum and ileum. Serum and urinary radioactivity was studied after administration of 10 μCi of [^{35}S]thiamine hydrochloride in 1-50 mg of non-radioactive thiamine hydrochloride.

2. Results suggest that intestinal absorption of thiamine hydrochloride is rate-limited. Though the results provide only indirect information on intestinal transport rates, they are consistent with the Michaelis-Menten relationship used to describe enzyme-substrate reactions. Calculations by this model yielded a V_{max} of 8.3 ± 2.4 mg and a K_m of 12.0 ± 2.4 mg for normal subjects with a significant decrease in V_{max} in malnourished alcoholics and a patient with resected small intestine.

3. Intestinal absorption and the calculated value of V_{max} for thiamine hydrochloride is increased in malnourished alcoholics after correction of malnutrition. These findings are consistent with the thesis that this vitamin is absorbed by a saturable mechanism and that the number of effective receptor sites may be reduced by malnutrition or intestinal resection.

Key words: thiamine hydrochloride absorption, malnutrition, intestinal resection.

Thiamine deficiency syndromes may occur despite oral intake of established minimum daily requirements of thiamine hydrochloride (Fennelly, Frank, Baker & Leevy, 1964). This is largely due to malabsorption of this vitamin, resulting from tissue changes induced by malnutrition (Thomson, Baker & Leevy, 1968), ethanol toxicity (Thomson, Baker & Leevy, 1970) or other factors. It is widely believed that interference with a rate-limited process may be responsible for defective absorption of thiamine hydrochloride. This view is supported by a decrease in absorption of [^{35}S]thiamine hydrochloride in patients with coeliac disease, which

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returned to normal following a gluten-free diet (Thomson, 1966a). A similar decrease in thiamine hydrochloride absorption occurs in malnourished alcoholics, which also returns to normal with correction of nutritional deficiency (Thomson *et al.*, 1970).

Nevertheless, several workers have failed to demonstrate a rate-limited process for thiamine hydrochloride absorption in animals (Turner & Hughes, 1962; Spencer & Bow, 1964), although others claim to have demonstrated a saturable mechanism (Ventura, Ferrari, Thalidac & Rindi, 1969; Friedman, Knieciak, Keegan & Sheft, 1948; Morrison & Campbell, 1960). Moreover, the validity of conclusions on studies of thiamine absorption in man has been questioned because of difficulty in identifying metabolites, and because of lack of information on intestinal breakdown, storage and utilization of the administered vitamin (Thomson, 1969). Absorption tests with [^{35}S]thiamine hydrochloride indicate that the administered radioactivity is absorbed and excreted with negligible breakdown of the vitamin. This substance was used in the present studies to evaluate the kinetics of intestinal transport of thiamine hydrochloride.

MATERIALS AND METHODS

Radioactive material

[^{35}S]Thiamine hydrochloride was obtained from The Radiochemical Centre, Amersham, Bucks., U.K. It had a specific radioactivity of 356 mCi/g and was found to be radiochemically pure when tested by descending chromatography on Whatman No. 1 paper using *n*-propanol-water-1 M-sodium acetate buffer, pH 5 (7:2:1, by vol.) and by thin layer chromatography on silica gel using pyridine-acetic acid-water (10:1:40, by vol.). The solid material was dissolved in distilled water, 100 μCi was dispensed into each ampoule, freeze dried, and stored at -20°C . It was reconstituted by adding 20 ml of water and non-radioactive thiamine hydrochloride so that each test dose contained from 1.0–50 mg of thiamine hydrochloride and 10 μCi of radioactivity.

Studies on patients

The absorption of [^{35}S]thiamine hydrochloride was studied by previously described methods (Thomson, 1966a, b). Less than 10% of the thiamine was broken down before absorption or during passage through the body (Thomson, 1966a). Informed consent was obtained from both patients and normal subjects to the investigation of thiamine metabolism. After an overnight fast patients received a parenteral injection of 200 mg of non-radioactive thiamine hydrochloride, immediately after which the radioactive material was given orally. Arterial blood was collected via an indwelling catheter at 0, 3, 6, 10, 20, 30, 40, 60, 90, 120, 150 and 180 min. Urine was collected hourly for the first 5 h and then after 12, 24, 48 and 72 h. Blood and urine radioactivity were determined in a Packard Tri-Carb liquid-scintillation counter, with 0.8 g/100 ml of 2,5-diphenyloxazole and 5 mg/100 ml of 1,4-bis-(5-phenyloxazol-2-yl)benzene dissolved in toluene as the liquid scintillator. The samples were prepared by adding 1.0 ml of urine or serum to 3 ml of methanolic 1.5 M-Hyaminate chloride, and 10 ml of liquid scintillator; 10 nCi of the test dose was added as an internal standard. The accuracy of the counting of radioactivity in the urine was determined by counting duplicate samples at three different concentrations of radioactivity. The following results were obtained: 8.3 ± 0.1 , 8.3 ± 0.1 nCi/ml; 0.76 ± 0.1 , 0.76 ± 0.1 nCi/ml; 0.0408 ± 0.00028 , 0.0406 ± 0.0003 nCi/ml. Tests were undertaken to determine the effectiveness of the 200 mg flushing dose of non-radioactive

thiamine hydrochloride when larger oral doses of radioactive thiamine were tested. Twenty-four healthy subjects were given either 1.0, 5.0 or 20.0 mg of radioactive thiamine orally together with 200 mg of non-radioactive thiamine intravenously. The results were compared with those obtained in the same subject, given the same test as before, but with additional intravenous injections of 100 mg of non-radioactive thiamine 4 h, 9 h, 12 h and 24 h after the oral dose. Thirteen malnourished alcoholics were given the same tests using 5.0 mg of oral radioactive thiamine.

Absorption studies were conducted in sixty healthy subjects (ages 28–80; twelve females), twelve chronic alcoholics with clinical and laboratory evidence of thiamine deficiency, and a patient with resection of the ileum and jejunum (except for 10 cm). Six of the patients with thiamine deficiency had abducens palsy and evidence of Wernicke's encephalopathy, and the other six had peripheral neuropathy. Each of the patients had a significantly decreased blood thiamine concentration as measured by the *Ochromonas danica* technique (Baker & Frank, 1968). Intestinal biopsies obtained from the alcoholic patients appeared normal under light microscopy. A biopsy taken from the proximal end of the anastomosed intestine in the patient with a resected small intestine also revealed a normal villous pattern. Five healthy subjects were given 20–100 mg of non-radioactive thiamine 0.5–3 h before giving the 1.0 mg radioactive oral dose together with the 200 mg intravenous flushing dose.

The normal subjects, two alcoholics and the patient with intestinal resection were each given three different dose levels of thiamine orally, the order in which the various doses were given being randomized. Urine was collected in 0–5, 5–12, 12–24, 24–48 and 48–72 h samples.

Calculations

The Michaelis–Menten formulation (Michaelis & Menten, 1913) was used to calculate maximum removal rate (V_{max}) by replacing reaction velocity and substrate concentrations in the original equation by thiamine excretion (v) and dose (d), respectively.

$$v = \frac{V_{max} \cdot d}{K_m + d} \quad (1)$$

Regression analysis was performed to test the applicability of this formula to our data and to obtain the V_{max} and the Michaelis constant (K_m). For this d was treated as the independent variable and v as a normal randomly distributed variable with the expected value:

$$E(v/d) = \frac{V_{max} \cdot d}{K_m + d} \quad (2)$$

and the variance:

$$\text{Var}(v/d) = \sigma^2 \quad (3)$$

Two or more elimination rates were determined using the same dose of thiamine hydrochloride to calculate confidence and estimate variation around the regression line. The 95% confidence limits were established for regression lines at their origin. The non-logarithmic transformation of Lineweaver & Burk (1934) was used to calculate the V_{max} and K_m . The least-squares procedure of Wilkinson (1961) was used to obtain the standard errors of the intercepts of the plot.

RESULTS

Normal subjects excreted $33.0 \pm 2.7\%$ (SEM) of a 5.0 mg oral dose of [^{35}S]thiamine hydrochloride whereas malnourished alcoholics excreted $14.6 \pm 3.2\%$ (SEM) and the patient with intestinal resection 8.5% of the oral dose (Tables 1 and 2). After a 6-8 week period of a nutri-

TABLE 1. Thiamine absorption in normal subjects and malnourished alcoholics. Results are expressed as the mean % of the oral dose \pm SEM. Subjects were given 5.0 mg of [^{35}S]thiamine hydrochloride orally together with 200 mg of intravenous non-radioactive dose. The loading was an additional 100 mg of intravenous non-radioactive thiamine hydrochloride at 4, 9, 12 and 24 h. The treated alcoholics were given 6-8 weeks of a nutritious diet with vitamin supplements.

Subjects	Loading	
	Before	After
Normal	33.0 ± 2.7	35.3 ± 2.1
Alcoholics:		
(a) Untreated	14.6 ± 3.2	16.3 ± 5.7
(b) Treated	39.4 ± 3.5	43.1 ± 4.2

TABLE 2. The 72 h cumulative urinary radioactivity in normal subjects and a patient with intestinal resection. Results are expressed as % of the oral dose.

Subjects	Oral dose of [^{35}S]thiamine hydrochloride (mg)		
	1.0	5.0	20.0
Normal			
Mean	51.0	33.0	25.9
SEM	2.1	2.7	2.6
Intestinal resection*			
Mean	13.2	8.5	5.5

* Duodenum, 10 cm of jejunum and descending colon only remaining.

tious diet and vitamin supplements, alcoholics excreted $39.4 \pm 3.5\%$ of the administered radioactive thiamine. Additional intravenous flushing doses of non-radioactive thiamine did not increase the excretion of radioactivity in healthy subjects (Fig. 1). A one-way analysis of variance computed on the difference between the two tests showed that extra flushing doses did not significantly alter the excretion in healthy subjects [$F(1, 22) = 0.704$; degrees of freedom (2, 22)] = 0.99) or in malnourished alcoholics (mean in standard test = $14.6 \pm 3.2\%$; with additional flushing = $16.3 \pm 5.7\%$). When two groups of healthy subjects of mean ages 49 years and 82 years were compared using regression analysis at three different dose levels no correlation

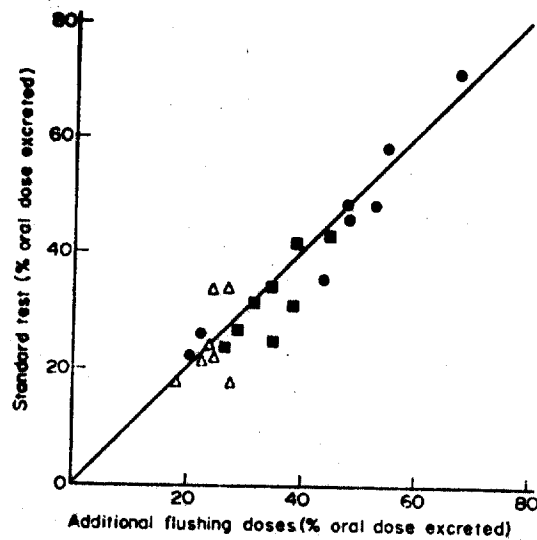


FIG. 1. Effect of giving additional intravenous flushing doses to control subjects receiving 1.0 mg (●), 5.0 mg (■) or 20 mg (Δ) of radioactive thiamine orally.

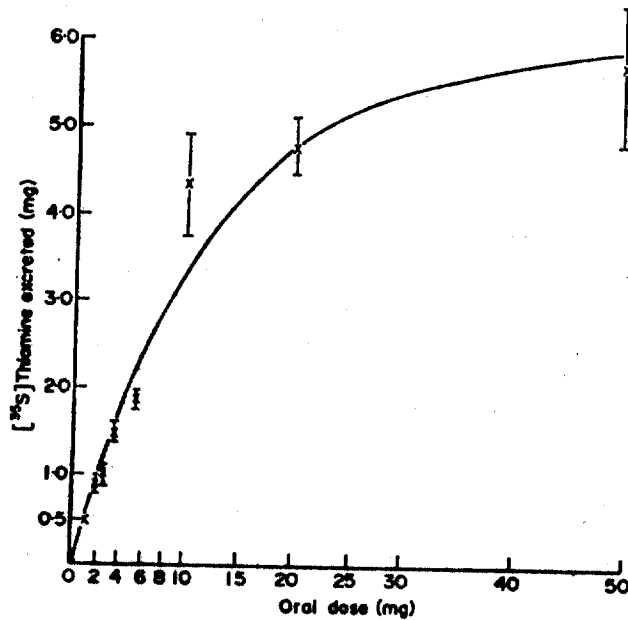


FIG. 2. Relationship between the dose of radioactive thiamine given orally and the cumulative 72 h urine radioactivity. Each point represents a mean value and the standard error is indicated. 200 mg of non-radioactive thiamine hydrochloride was given intravenously with each oral dose.

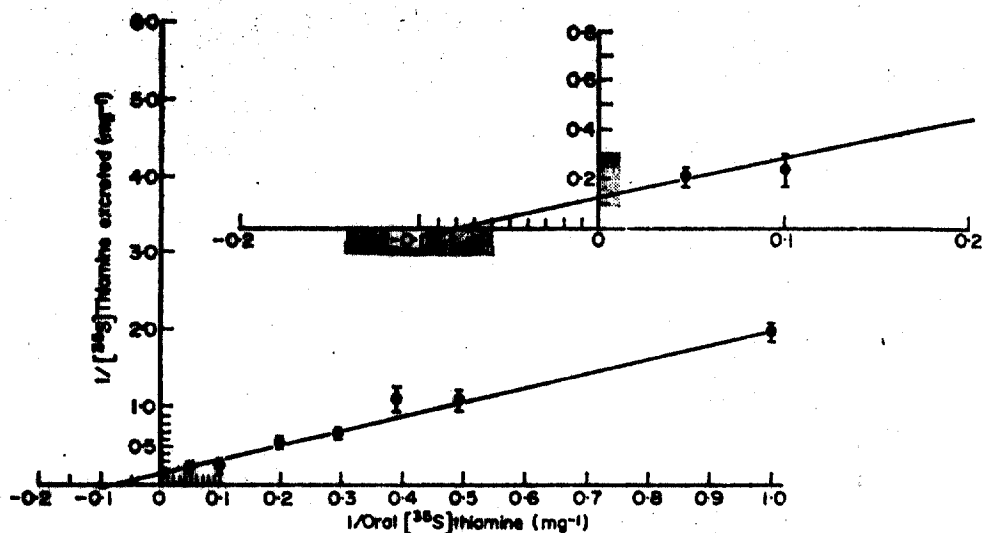


FIG. 3. Linear relationship between the reciprocal of the dose of radioactive thiamine given orally and the reciprocal of the cumulative 72 h urinary radioactivity. Each point represents a mean value; 200 mg of non-radioactive thiamine hydrochloride was given intravenously with each oral dose. The insert shows the values of V_{max} and $K_m \pm$ two standard errors of the mean.

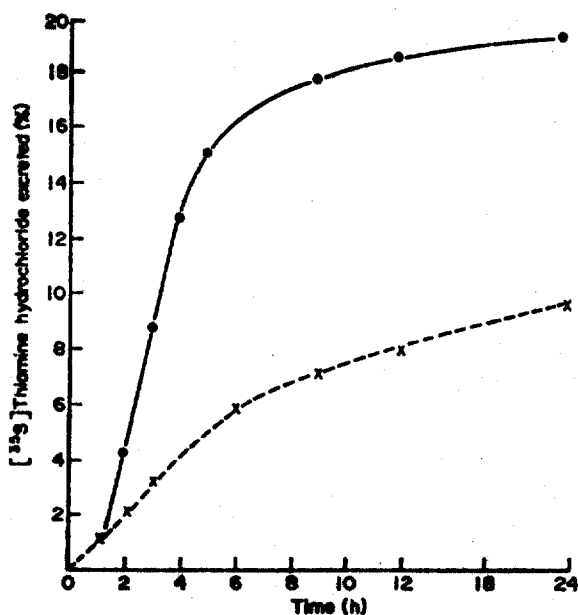


FIG. 4. Cumulative urinary excretion of radioactivity after 20 mg of $[^{35}\text{S}]$ thiamine hydrochloride was given orally together with a dose of 200 mg of thiamine hydrochloride intravenous non-radioactive flushing material (●). The test is repeated on the same subject 0.5 h after a 100 mg dose of non-radioactive thiamine hydrochloride was given orally (x).

between age and percentage of excretion of radioactivity could be demonstrated [At 1.0 mg oral dose/age $t = 0.002$ (35 degrees of freedom) $P > 0.5$; 5.0 mg, $t = 0.687$ (27 degrees of freedom) $P = 0.5$; 20 mg, $t = 0.673$ (30 degrees of freedom) $P > 0.5$].

The relationship between the administered and excreted radioactive thiamine in subjects receiving eight different dose levels from 1.0 to 50.0 mg of thiamine hydrochloride provides evidence of saturation (Fig. 2). A typical Michaelis-Menten curve fits the data if one excludes

TABLE 3. Urinary excretion of radioactivity in normal subjects with and without a prior non-radioactive oral loading dose. In the standard test the patient was given 20 mg of [35 S]thiamine hydrochloride orally together with 200 mg of non-radioactive thiamine intravenously. An additional 100 mg of non-radioactive thiamine was given 1 h later. The urine was collected for 72 h.

Subject	Standard test (% of oral dose excreted)	Standard test with prior non-radio- active oral dose*		
		Non-radioactive oral dose		% of oral dose excreted
		Time before (h)	Amount (mg)	
E.C.	43.6	0.5	20	28.0
T.M.	40.7	1.0	20	24.8
P.H.	19.8	0.5	100	12.7
T.L.	21.8	1.0	100	11.5
N.B.	18.6	3.0	100	8.9

* Test performed as above in the same patient but 20 mg or 100 mg of non-radioactive thiamine hydrochloride was given orally 0.5-3 h before the radioactive dose.

thiamine excretion values after the 10 mg dose of thiamine hydrochloride. A straight-line relationship in which the intercepts on X and Y axes are significantly different from zero was obtained when the data was plotted by the method of Lineweaver & Burk (1934). The mean calculated maximum amount of thiamine (V_{max}) absorbed after a single oral dose was 8.3 ± 2.4 mg in healthy subjects and well-nourished alcoholics. The size of the oral dose (K_m) required to produce half-maximum absorption was 12.0 ± 2.4 mg when oral doses greater than twice this dose were given, and the amount excreted did not exceed the maximum predicted value when the 50 mg dose response is excluded (Fig. 3). Values were obtained by use of the weighted least-squares procedure (Wilkinson, 1961). Oral administration of 20-100 mg of non-radioactive thiamine to subjects 0.5-3 h before the 1.0 mg radioactive oral dose produced up to 50% decrease in the absorption of the radioactive dose (Table 3; Fig. 4).

Malnourished alcoholics and the patient with intestinal resection showed a considerable decrease in (a) serum of radioactivity amounts; (b) rate of rise in cumulative urinary excretion; (c) total amount of radioactivity excreted at all three dose values (Figs. 5 and 6). The Lineweaver-Burk plot was linear again (Fig. 7). The patient with intestinal resection had a V_{max} of 1.1 mg. Assuming absorption of thiamine hydrochloride in alcoholic subjects still conforms

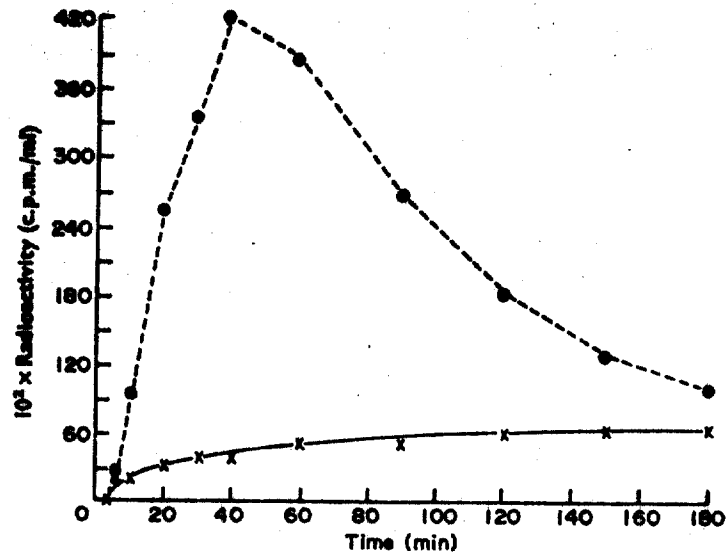


FIG. 5. The patterns of serum radioactivity seen in a normal subject (●) and a patient with an intestinal resection (x) after 1.0 mg of radioactive thiamine was given orally and a 200 mg flushing dose of non-radioactive thiamine hydrochloride.

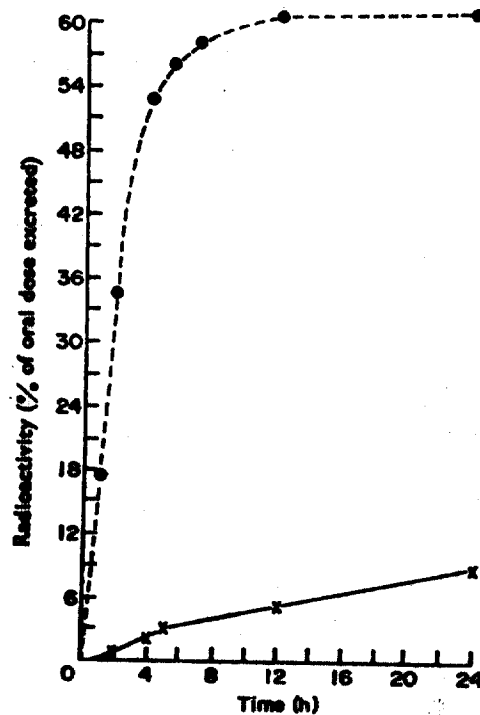


FIG. 6. Cumulative urinary excretion of radioactivity after 1.0 mg of radioactive thiamine was given orally and a 200 mg flushing dose of non-radioactive thiamine hydrochloride in a normal subject (●) and a patient with intestinal resection (x).

to Michaelis-Menten kinetics, thereby allowing use of three points serial study, a malnourished subject with fatty liver showed a V_{max} of 1.5 mg on admission; this value had increased to 8.0 mg after a 6 week period on a nutritious diet. In contrast with the marked decrease in

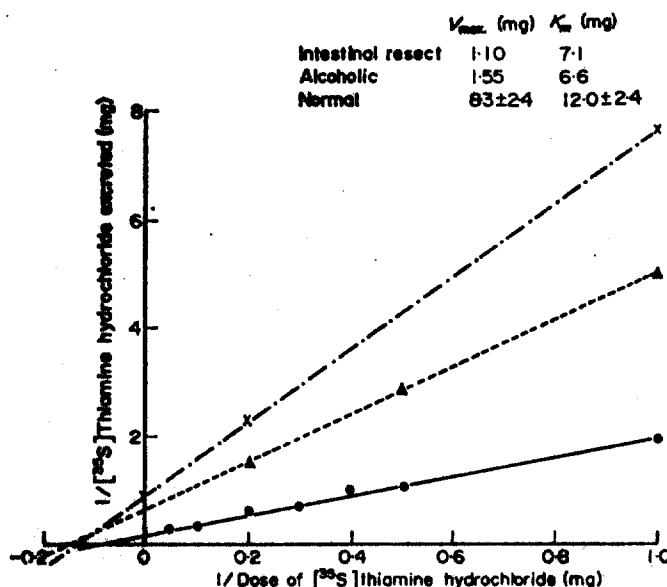


FIG. 7. Linear relationship between the reciprocal of the dose of radioactive thiamine given orally and the reciprocal of the cumulative 72 h urinary radioactivity in normal subjects (●) a malnourished alcoholic (Δ), and a patient with intestinal resection (×).

V_{max} in the malnourished alcoholic and the patient with intestinal resection, the K_m was not significantly altered, the K_m intercept for these patients falling within two standard errors of the intercept for healthy control subjects.

DISCUSSION

Our data indicate that in normal man there is a maximum absorption of approx. 8.3 mg of thiamine hydrochloride after ingesting a single dose of this compound. Other workers have suggested that there is an upper limit to the absorption of thiamine in man. However, this view has been based on measurements of urinary excretion of thiamine without identification of metabolites or evaluation of diversion of administered thiamine to other areas (Friedman *et al.*, 1948; Morrison & Campbell, 1960). The use of radioactive thiamine in conjunction with a flushing dose of non-radioactive thiamine increases the reliability of the present observations. Additional intravenous flushing doses did not increase the excretion of radioactivity or alter the pattern of excretion in malnourished alcoholic patients or normal subjects (Fig. 1) and when labelled thiamine was given intravenously with the flushing dose, approximately 80% of the label was excreted within 4 h and about 90% within 24 h (Thomson, 1969). The amount of total body thiamine has been estimated to be 30 mg (Takeda, 1947) and the assumption that a large intravenous dose will allow practically all of any additional thiamine which may be

absorbed to be excreted in the urine appears to be well supported. Simultaneous measurements of urinary and faecal excretion of orally administered labelled thiamine given with a flushing dose show an inverse relationship (Tomassulo, Kater & Iber, 1968).

The maximum ability of the kidney to excrete thiamine was not exceeded during the tests (Thomson *et al.*, 1970) as is evident from the fact there was no significant change in the serum pattern of radioactivity in the presence of the additional flushing doses which could influence saturation. Absorption tests in the same subject before and after treatment show very similar patterns of serum and urinary radioactivity which differ only in the amount of radioactivity present at any one time. However, neither the comparative facility with which thiamine hydrochloride is absorbed from different areas of the intestine, nor the influence of intestinal motility on absorption is known. An earlier radioactive peak might have been expected had there been increased motility which altered absorption.

If one accepts the difficulties in quantifying the influence of multiple biological systems and the variable effects of flushing doses, the relationship between the dose of thiamine given and the amount recovered from the urine obeys Michaelis-Menten kinetics, suggesting that a rate-limiting step is involved. It has been assumed that thiamine hydrochloride is phosphorylated during its absorption (Linneweh & Muller, 1940; Tauber, 1937; Cerecedo, Eich & Bresnick, 1954; Rindi, Ventura, De Guiseppe & Sciorelli, 1966); however, this step does not account for the observed saturable phenomenon, since absorption of thiamine propyl disulphide which is also phosphorylated during intestinal transport is not rate-limited (Thomson, Frank, Baker & Leevy, 1971).

The suggestion that thiamine absorption follows Michaelis-Menten kinetics makes it possible to ascertain the mechanism whereby various factors interfere with its intestinal transport. The K_m value reflects the affinity of the transport system for thiamine and is equal to the dose of thiamine which is removed from the intestine at half maximum removal rate. An evaluation of the effects of disease on K_m and V_{max} values may be used to quantify and characterize the mechanism responsible for the decreased intestinal absorption. Both the patient with the intestinal resection and the alcoholic patients showed a marked decrease in V_{max} without a significant decrease in K_m . This is found in non-competitive inhibition and is consistent with the hypothesis that there has been a decrease in the number of receptor sites available. The reversible decrease in V_{max} noted in the malnourished alcoholics suggests that receptor sites are temporarily damaged in these instances. The time-lapses required for restoration of normal thiamine absorption are attributable to the need for repair or regeneration of mucosal cells with adequate receptor sites.

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Alpha-Glycerophosphate and Lactic Dehydrogenase Activities in Tissues of Thiamine-Deficient Rats¹

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At present it is difficult to interpret the thiamine deficiency syndrome entirely in terms of the biochemical defect of a decrease in thiamine pyrophosphate. The severity of the metabolic derangement and the peculiar tissue predilection associated with the deficiency are not in agreement with the degree of diminution in activity of such enzymes as pyruvic oxidase. In fact, despite considerable decrease in activity of this enzyme in induced deficiency, there remains activity almost adequate to meet the physiologic demands (Shils et al., '41). It has further been reported that the decrease is as much a loss of apoenzyme as of coenzyme (Oshima et al., '60) and that there is little change in the ability of the thiamine-deficient rat to oxidize C¹⁴-labeled pyruvic and lactic acids (Jones and de Angelis, '60).

Similarly one can question that the decrease in activity of other thiamine pyrophosphate dependent enzymes (such as α -ketoglutaric dehydrogenase and transketolase) is primarily responsible for observed specific biochemical lesions. There are observations which cannot, at present, be interpreted on the basis of a decrease in thiamine pyrophosphate concentrations. Among these are the accumulation of methylglutamate as a consequence of vitamin B₁₂ deficiency, which has been consistently reported (Salem, '54, '55), and the discrepancy in the deficiency syndromes provoked by erythiamine or pyrithiamine. These latter differences prompted Woolley and Merrifield ('54) to propose a new role of thiamine beyond the presently known functions.

Recently a new thiamine derivative, thiamic acid, was isolated from the diphosphopyridine nucleotide (DPN)-dependent crystalline rabbit muscle α -glycerophos-

phate dehydrogenase.³ Although the enzymatic function of this compound is not clear, it seemed of interest to investigate the effect of thiamine deficiency on this and other dehydrogenases. This paper describes the results of this investigation.

EXPERIMENTAL

Female Wistar rats with initial weights of around 100 gm were fed a commercial thiamine-deficient diet.⁴ Thiamine and thiamine antimetabolites were administered by inclusion of the compounds in the drinking water, which was changed daily and supplied *ad libitum*.

To obtain material for enzymatic assay, the rats were decapitated and the tissues quickly excised and frozen until assay. When required, the organs were homogenized in a Potter-Elvehjem homogenizer in ice cold 0.154 M KCl. For muscle, a 10% homogenate was used, whereas for liver, a 20% homogenate was prepared. The latter was diluted ten-fold before assay.

α -Glycerophosphate dehydrogenase activity was assayed by following spectrophotometrically the reduction of the pyridine nucleotide in the following system in a final volume of 3.0 ml: disodium-DL- α -glycerophosphate, 20 μ moles; DPN, 2 μ moles; mercaptoethanol, 10 μ moles; and tris(hydroxymethyl)-aminomethane buffer, pH 9.3, 300 μ moles. The reaction was initiated with enzyme. Lactic dehydrogenase was assayed similarly, but with the

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¹ Supported by grant G-5833 of the National Science Foundation.

² Investigator in the Howard Hughes Medical Institute.

³ van Eys, J. 1960 Thiamic acid, the non-protein component of α -glycerophosphate dehydrogenase. *Federation Proc.* 19: 25 (abstract).

⁴ Nutritional Biochemicals Corporation, Cleveland.

omission of neurospiroethanol and substitution of *Neurospora crassa* for the glycerophosphate. The both organisms are tests of sensitivity defined as an increase in optical density of 0.001 per minute under the conditions specified. Alcohol was assayed chemically (Taylor, '58).

Protein was estimated with the biuret test (Weichselbaum, '46), using crystalline ovalbumin as standard.

RESULTS

Growth of the experimental groups. Thiamine deficiency was induced by three devices: (1) omission of the vitamin from

the diet, or by administration of either (2) oxythiamine or (3) pyriethiamine. The antimetabolites were fed in conjunction with a thiamine fraction equivalent to half the amount required to counteract the level of antimetabolites: ratios of 50/1 for oxythiamine to thiamine and 3/1 for pyriethiamine to thiamine. This design was adopted to avoid a purely dietary deficiency in the rats fed the antimetabolites. In figure 1 is shown the growth obtained with the three levels of antimetabolites. At the higher level oxythiamine appears to be the more effective growth inhibitor, even though the effect at low concentrations of

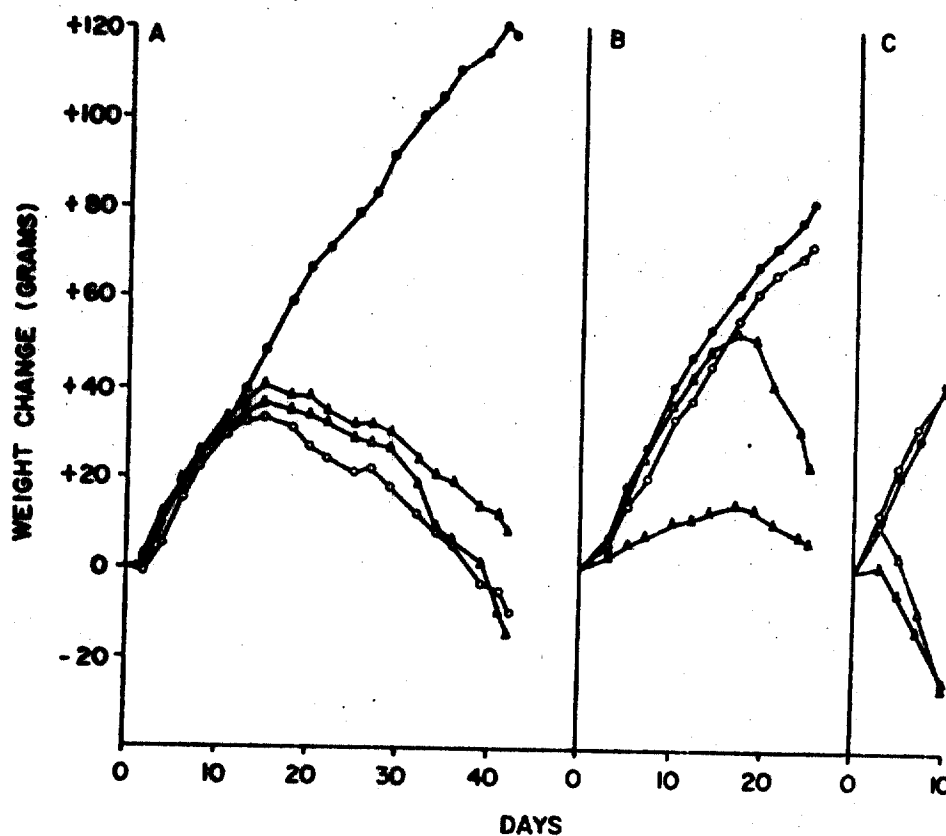


Fig. 1 Growth curve of thiamine-deficient animals treated with antimetabolites. Code: solid circles, control; open circles, animals receiving the dose of thiamine of the treated groups; closed triangles, oxythiamine-treated; open triangles, pyriethiamine-treated.

Group A Five subgroups of oxythiamine plus 0.1 μ g of thiamine or 0.3 μ g of pyriethiamine plus 0.1 μ g of thiamine administered per milliliter of drinking water.

Group B Fifty subgroups of oxythiamine plus 1 μ g of thiamine or 3 μ g of pyriethiamine plus 1 μ g of thiamine administered per milliliter of drinking water.

Group C Three subgroups of oxythiamine plus 5 μ g of thiamine or 10 μ g of pyriethiamine plus 5 μ g of thiamine administered per milliliter of drinking water.

Control animals received at all times 10 μ g of thiamine per milliliter of drinking water.

oxythiamine and pyriothiamine are comparable. Oxythiamine never produced polyneuritis, however, whereas pyriothiamine did so at all levels. This was true even when the symptoms were produced acutely (table 1) and is in agreement with observations in the mouse (Eusebi and Casacchia, '49).

α -Glycerophosphate dehydrogenase activity. The level of α -glycerophosphate dehydrogenase activity dropped markedly as a consequence of thiamine deficiency in skeletal muscle, liver and brain. The level of the enzyme in heart is too low to measure accurately. Some results obtained in dietary deficiency are shown in table 2.

The effect was not due to the diminished food intake of the thiamine-deficient animals, since paired feeding did not result in lowered activity of the enzyme. The decrease in activity is a relatively late effect, since animals sacrificed at the initial onset of weight loss did not show it.

Additional evidence that this decrease is a reflection of thiamine nutriture was that animals made deficient in 7 days with antimetabolites showed a similar decrease in activity (table 3). The effect of oxythiamine at lower concentrations was less than that occurring in either dietary- or pyriothiamine-induced deficiency. This is in sharp contrast with the effect on lactic dehydrogenase, as will be described later.

The decrease in activity was proportional to the level of either antimetabolite fed (table 3), and was not associated with a

concomitant loss in liver protein concentration (table 2). The decrease did coincide with a marked loss in liver weight so that, expressed on a basis of total liver, there is an even more striking difference.

Lactic dehydrogenase activity. The lactic dehydrogenase activity also decreased in thiamine deficiency, but not as strikingly as the α -glycerophosphate dehydrogenase activity (30 vs. 50%). Peculiarly enough, however, even at low oxythiamine levels, which had little effect on α -glycerophosphate dehydrogenase activity, the lactic dehydrogenase activity decreased to barely detectable levels. (In liver, 20 units per mg is barely detectable.) Also in contrast with the α -glycerophosphate dehydrogenase activity, lactic dehydrogenase was lower in oxythiamine-treated rats than in rats treated with equivalent amounts of pyriothiamine. These points are also illustrated in table 3.

The effect of starvation on dehydrogenase activity. Although paired feeding did not abolish the effect of thiamine deficiency on these enzyme levels, it has been reported (Weber, '60) that lactic dehydrogenase decreases in starvation. To determine the magnitude of this effect, rats weighing about 250 gm were starved for 5 or 10 days. Control animals were fed a commercial laboratory chow.⁵ The lactic dehydrogenase of the liver decreased about 30% (table 4), in agreement with the ob-

⁵ Purina Laboratory Chow, Ralston Purina Company, St. Louis.

TABLE 1
Incidence of symptoms in thiamine-deficient rats

Group	Series	Days fed diet	Thiamine administered	Antimetabolite administered	No. rats/group	Incidence of polyneuritis ^a	No. of deaths ^b
			$\mu\text{g/ml}$ drinking water	$\mu\text{g/ml}$ drinking water			
Control	600 ^c	42	10	—	9	0	0
Restricted			0.1	—	9	5	2
Oxythiamine			0.1	5	9	0	0
Pyriothiamine			0.1	0.2	9	8	4
Control	800 ^c	7	10	—	10	0	0
Restricted			5	—	3	0	0
Oxythiamine			5	250	9	0	1
Pyriothiamine			5	10	9	9	3

^a The numbers designate the incidence out of the total.

^b Group A in figure 1.

^c Group C in figure 1.

TABLE 2
α-Glycerophosphate dehydrogenase activity of distended thiamine-deficient rats¹

Group	No. animals	Weight change gm	Liver			Muscle		
			Weight gm	Protein mg/mg liver	α-GDP dehydrogenase units/mg liver	Protein mg/mg muscle	α-GDP dehydrogenase units/mg muscle	Protein
Control, all Medium	0	+100% ± 12.6	—	0.20 ± 0.005	120 ^a ± 70.0	0.053 ± 0.013	—	—
Pair-fed control	11	+14 ^b ± 17.5	—	0.37 ± 0.0045	79 ^a ± 33.2	0.044 ± 0.008	—	—
Deficient	11	-15 ± 14.4	—	0.20 ± 0.009	46 ± 35.3	0.008 ± 0.010	—	—
Pair-fed control	8	+14 ^b ± 20.3	0.2 ^c ± 0.96	0.20 ± 0.004	69 ^a ± 32.2	—	—	—
Deficient	8	-34 ± 9.5	4.0 ± 0.45	0.33 ± 0.044	37 ± 19.1	—	—	—
Control, all Medium	9	+118 ^b ± 19.0	8.4 ^c ± 1.05	0.24 ± 0.046	57 ^a ± 13.6	—	—	—
Deficient	7	-9.5 ± 25.3	3.6 ± 0.71	0.35 ± 0.046	27.5 ± 10.7	—	—	—

¹ Figures indicate ± standard deviation.

² Pair-fed animals are compared in paired variance.

³ Difference statistically significant from the deficient group ($P < 0.01$).

⁴ Difference statistically significant from pair-fed control group ($P < 0.01$).

⁵ Difference statistically significant from the deficient group ($P < 0.05$).

servations reported by Weber. A similar drop occurred in α -glycerophosphate dehydrogenase but in neither case was the decrease as great as that occurring in thiamine deficiency (table 4).

DISCUSSION

Many factors enter into the interpretation of studies such as these. Although paired feeding equalized the food intake between deficient and nondeficient groups, it does not necessarily equalize the food utilization. In fact, thiamine-deficient animals almost always lose more weight than their pair-fed controls (table 2). One may postulate that the effects of thiamine deficiency include a "metabolic starvation." This interpretation cannot be disproven, but the effect of high levels of thiamine antimetabolites, where the weight loss is less than in acute starvation but the enzymatic effects are greater, argues in favor of some specific thiamine effect. Other points are in favor of this concept. A decrease in serum lactic dehydrogenase in humans after pyridoxamine treatment has been reported (Wendel, '60), yet these subjects were not acutely deficient. Several enzymes are not affected by thiamine deficiency (Terroine, '60). Furthermore, protein biosynthesis is possible in extreme thiamine deficiency as evidenced by the unimpaired ability of deficient animals to make antibodies (Axelrod and Hopper, '60). Also in this study aldolase was found to be unaffected.

The peculiar differential effect between oxythiamine on the one hand and pyridoxamine and dietary deficiency on the other, which was seen in this investigation, may well be a metabolic basis for the difference in the clinical syndrome which results from these forms of deficiency. It is tempting to speculate that the difference is partially the result of a differential effect of these antimetabolites on thiamic acid levels or activity. Such interpretations must await further investigation of the role of this compound. The data presented here do not shed light on the function of thiamic acid on α -glycerophosphate dehydrogenase. It has not been possible to activate a deficient muscle with a supernatant from a control muscle.

TABLE 3
Effect of oxythiamine and pyriethiamine on dehydrogenase activities¹

Dietary history	No. animals	Anti-metabolite administered ²	Weight change at sacrifice ³	Liver α -glycerophosphate dehydrogenase ⁴	Liver lactic dehydrogenase
		$\mu\text{g/ml}$ drinking water	gm	units/mg liver	units/mg liver
Control, ⁴ ad libitum	9	—	+119 \pm 19.0	57 \pm 13.6	53 \pm 10.8
Deficient	7	—	— 9.5 \pm 25.3	27.5 \pm 12.2	37 \pm 5.0 ⁵
Oxythiamine	9	5	+ 9 \pm 17.7	44.5 \pm 11.6 ^{5,6}	23.5 \pm 4.5 ⁷
	6	50	+ 6.5 \pm 12.5	37 \pm 5.5	24.5 \pm 3.1 ⁸
	6	250	— 24 \pm 4.7	27 \pm 9.1	19 \pm 4.1 ⁹
Pyriethiamine	5	0.2	— 17 \pm 8.3	39 \pm 12.2	—
	6	2	+ 27 \pm 13.2	33 \pm 9.5	27.2 \pm 7.35 ⁸
	6	10	— 25 \pm 8.0	26 \pm 11.3	25 \pm 6.5 ⁹

¹ Figures indicate \pm standard deviation.

² The three levels of anti-metabolites correspond to the series described in figure 1.

³ All figures in these columns are significantly different from the control group at the $P < 0.01$ level except α -glycerophosphate dehydrogenase activity at 5 μg of oxythiamine where the significance is at the $P < 0.05$ level.

⁴ The control group is representative of all the control groups included with each level of oxythiamine and pyriethiamine.

⁵ Significantly different from the control group at a level of $P < 0.05$.

⁶ Significantly different from the control group at a level of $P < 0.01$.

⁷ Significantly different from the dietary deficient group at a level of $P < 0.02$.

⁸ Significantly different from the dietary deficient group at a level of $P < 0.01$.

TABLE 4
Effect of complete starvation on dehydrogenase activities¹

	No. animals	Weight change	Liver weight	Liver protein	Liver lactic dehydrogenase	Liver α -glycerophosphate dehydrogenase
		gm	gm	mg/mg liver	units/mg liver	units/mg liver
Control	9	+18.4 \pm 5.15	8.4 \pm 0.98	0.43 \pm 0.089	32 \pm 7.3	45 \pm 8.8
Starvation, 5 days	9	-46.1 ¹ \pm 5.09	4.9 ² \pm 0.56	0.36 \pm 0.068	23 \pm 10.3	30 ³ \pm 8.0
Starvation, 10 days	4	-82.3 ^{1,2} \pm 9.15	3.9 ^{1,2} \pm 0.21	0.41 \pm 0.050	28 \pm 6.5	32 ⁴ \pm 7.6

¹ Figures indicate \pm standard deviation.

² Significantly different from control at a level of $P < 0.01$.

³ Significantly different from short starvation at a level of $P < 0.01$.

⁴ Significantly different from control at a level of $P < 0.05$.

SUMMARY

Thiamine-deficient rats have a striking decrease in liver and muscle α -glycerophosphate dehydrogenase activity. When the deficiency was induced with oxythiamine or pyriethiamine the decrease in enzyme was proportional to the dose administered. At equivalent levels, oxythiamine was less effective than pyriethiamine.

For lactic dehydrogenase the decrease in activity was less pronounced in dietary

deficiency but oxythiamine was more effective in lowering this enzyme concentration.

It is concluded that this represents a true effect of thiamine deficiency and not of starvation. The results are discussed in the light of the recently discovered thiamic acid.

ACKNOWLEDGMENTS

The technical assistance of Mariene J. Greene and Russell Root is gratefully acknowledged.

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**A KIDNEY LESION ASSOCIATED WITH THIAMINE
FEEDING EXPERIMENTS. J. van Eys & G. A. Elliott,*
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When Wistar rats are maintained on a thiamine deficient diet, supplemented with 10 µg/ml of thiamine in their drinking water, a kidney lesion develops in about 60% of the animals. The same diet, without thiamine, does not provoke such lesions. The pathology shows many eosinophilic tubular casts at the cortico-medullary junction. These casts are surrounded by a dense zone of mononuclear cells and eosinophils. The casts sometimes undergo calcification. When thiamine is replaced by 100 µg/ml. oxythiamine the lesions do not appear. However, when pyridoxamine is given in doses equivalent to oxythiamine (based on growth rate), the incidence of lesions approaches 100%. The rats are free of lesions when they are received from the dealer, and remain so on a stock chow. The lesions are not infectious in origin. The most reasonable hypothesis at present is that the lesions are caused by a breakdown product of thiamine in the drinking water. However, thiochrome is ineffective in provoking the lesions.

Metabolism of Thiamine-S³⁵ in the Rabbit.* (24091)

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McCarthy, Cerecedo, and Brown(1) showed that rats which had received an intramuscular injection of thiamine-S³⁵ excreted about 64% of the radiosulfur in urine, and between 1 and 2% in feces, within 10 days after administration. Isolation of thiamine-S³⁵ from urine by adsorption on synthetic zeolite Decalso showed that approximately 60% radiosulfur of the neutral sulfur fraction was contained in unchanged thiamine-S³⁵, and the remaining 40% was present in neutral sulfur compounds not adsorbed by Decalso. The present study was based on use of doses of radiovitamin that were in the physiological range, i.e., from 50 to 200 γ administered by oral and parenteral routes, to ascertain whether different routes of administration would lead to gross differences in the metabolic pattern.

Materials and methods. Metabolic studies were carried out using Chinchilla or Dutch breed adult rabbits. These were housed in wire-bottom metabolism cages allowing complete separation of urine and feces, and were given Purina Rabbit Chow and water *ad lib.* with supplement of lettuce and carrots once or twice weekly except immediately prior to and during experimental periods. Each individual experiment was carried out with 2 rabbits, and in so far as possible, the same pair of rabbits was used in successive experiments so that, in effect, each rabbit was its own control. Urine samples were collected in 10 ml of glacial acetic acid as a preservative which brought the pH from range 9-10 to 4-5, in which thiamine-S³⁵ and its metabolites would be more stable. Urine samples were filtered and diluted with distilled water. Feces were homogenized with 150 ml of water containing about 10 ml of concentrated hydrochloric acid. The homogenate stood several hours, was stirred occasionally, and finally centrifuged to re-

move the bulk of solid matter. The supernatant was filtered and diluted with distilled water. Radiosulfur of urine was determined by analysis of 3 sulfur fractions: inorganic sulfate, ethereal sulfate, and neutral sulfur. Fecal sulfur was partitioned into inorganic sulfate and neutral sulfur fractions. The method of Folin was utilized for isolation of inorganic sulfate, and total sulfate (inorganic plus ethereal) fractions, while the neutral sulfur fraction was obtained by subtracting total sulfate from total sulfur, as determined by the Denis modification of the method of Benedict (2a). All sulfate fractions were precipitated as barium sulfate, and filtered onto porous glass frits mounted in stainless steel holders. Precipitates were washed successively with water, methanol, and acetone, and dried at 110°C for one hour. Samples were counted in a Nuclear windowless or thin-window flow-gas Geiger-Mueller counter and scaler. All count rates were corrected for background, counter efficiency, decay of sulfur-35, and self-absorption. A sample of barium sulfate-S³⁵ was prepared from an amount of thiamine-S³⁵ identical to that administered to rabbits. This standard sample was counted each day that experimental samples were counted, and comparison of count rates of samples with this standard gave the percent recovery of the radiosulfur. In addition to sulfur analyses, urea excreted in urine was determined each day by the colorimetric method of Rosenthal, using diacetyl monoxime in arsenic acid(3). Under normal conditions of nutrition, daily excretion of urea parallels that of inorganic sulfate in urine(2b). Therefore urea : inorganic sulfate ratios were calculated daily to ascertain that no marked alterations occurred in nitrogen equilibrium. Preliminary experiments indicated that unchanged thiamine-S³⁵ and its thiazole-S³⁵ moiety were main metabolites of radiovitamin in rabbit urine, and procedures were devised to isolate these substances. A modification of the method of Herr(4) was used for isolation of thiamine-

* This investigation was aided in part by Contract between U. S. Atomic Energy Com. and Fordham University.

METABOLISM OF THIAMINE-S³⁵ IN THE RABBITTABLE I. Percent Recovery of S³⁵ in Urine and Feces of Rabbits after Administration of 100 γ of Thiamine-S³⁵ by Stomach Tube.

24-hr excretion period	Fraction	Urine		Feces	
		Rabbit 1	Rabbit 2	Rabbit 1	Rabbit 2
1st	Inorganic sulfate	2.00	1.29	2.10	.47
	Ethereal "	.92	1.37		
	Neutral sulfur	37.85	43.22	7.39	5.92
	Total	41.37	45.88	9.49	6.39
2nd	Inorganic sulfate	.74	.42	.57	.90
	Ethereal "	.42	.00		
	Neutral sulfur	3.26	2.28	2.37	1.35
	Total	4.42	2.70	2.94	2.25
3rd	Inorganic sulfate	.59	.42		
	Ethereal "	.11	.00		
	Neutral sulfur	2.99	.96		
	Total	3.69	1.38	1.48	.90
4th	Inorganic sulfate	.11	.21		
	Neutral sulfur	.88	.26		
	Total	.99	.47	.73	.55
5th	Total	.67	.21		
Total recovery		51.12	50.64	14.64	10.09

Total recovery, urine and feces: Rabbit 1, 65.76%; Rabbit 2, 60.73%.

S³⁵, in which it was removed from urine by adsorption on column of strong cation exchange resin Dowex-50-X8 with subsequent elution by concentrated hydrochloric acid. Recovery experiments indicated that between 80 and 90% of thiamine-S³⁵ could be removed from urine by this procedure. The hydrochloric acid eluate was evaporated to dryness over soda lime and phosphorus pentoxide in vacuum desiccator; the residue was taken up in about 10 ml of anhydrous methanol, the resulting solution filtered, and the filtrate evaporated to dryness. The final residue was dissolved in 5 to 8 ml of anhydrous methanol, and an aliquot of this solution was used for determination of the radiosulfur content. Thiazole-S³⁵ was extracted from urine with ether. Prior to extraction, about 25 mg of non-radioactive thiazole were added to urine to act as carrier, the urine adjusted to pH 10 by addition of sodium hydroxide, then extracted with ether in a continuous extractor for 12 to 16 hours. The ethereal extract was dried over anhydrous magnesium sulfate, and ether distilled off. The thiazole-S³⁵ residue was dissolved in 5 to 8 ml of anhydrous ethanol, and an aliquot used for determination of radiosulfur content. The presence of these metabolites in the extracts was confirmed by

paper chromatography on strips of Whatman #1 paper (1.5 x 22 inches) in the solvent system n-butanol-acetic acid-water (4 : 1 : 1). Ascending chromatography was used. The chromatograms were developed 8 to 10 hours in the solvent system, then dried at room temperature. Spots were identified by inspecting the chromatograms in ultraviolet light and by scanning the strips in a windowless flow-gas Geiger-Mueller counter designed for this purpose (Anderson Scannergram). Thiamine-S³⁵ bromide hydrobromide was prepared from thiourea-S³⁵(5).[†]

Results. Oral administration. In experiments in which thiamine-S³⁵ was administered to rabbits by oral route, 3 dose levels in the physiological range were used: 50, 100, and 200 γ . The thiamine-S³⁵ (about 25,000 counts/min/100 γ) was dissolved in 1 ml of water and administered to rabbits by stomach tube. The excretion pattern was the same for all 3 levels; however, total recovery of radiosulfur in urine varied from 50 to 90% in 4 to six 24-hour periods following administration of radiovitamin. In all cases, more than 50%

[†] We are indebted to Dr. Karl Folkers of Merck, Sharp, and Dohme, for 2-aceto-butyrolactone used in synthesis.

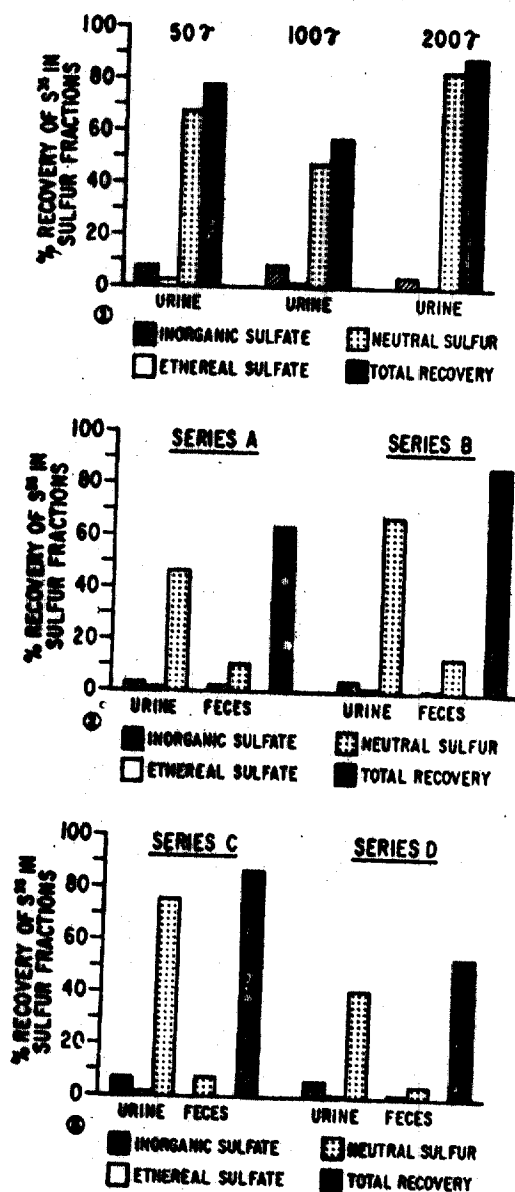


FIG. 1. Oral administration of thiamine- S^{35} to rabbits. Recovery of radiosulfur in urinary sulfur fractions (five 24-hr periods) after administration of 50, 100 and 200 γ of thiamine- S^{35} by stomach tube.

FIG. 2. Oral administration of thiamine- S^{35} to rabbits. Recovery of radiosulfur in urine and feces: Series A, 100 γ of thiamine- S^{35} by stomach tube; Series B, 100 γ of thiamine- S^{35} by stomach tube, followed 6 hr later by intramusc. inj. of 2 mg of non-radioactive thiamine. (Urine: five 24-hr periods; feces: four 24-hr periods.)

FIG. 3. Parenteral administration of thiamine- S^{35} to rabbits. Recovery of radiosulfur in urine and feces: Series C, 100 γ of thiamine- S^{35} by intramusc. inj.; Series D, 100 γ of thiamine- S^{35} by intrav. inj. (Urine: six 24-hr periods; feces: four 24-hr periods.)

of the recovered radiosulfur was in the neutral sulfur fraction of the urine, and the greater portion of this was recovered in urine excreted in the first 24-hour period. The second 24-hour period usually contained half, or less than half, of the amount of radiosulfur recovered in the first period, and again this was contained primarily in the neutral sulfur fraction. Radiosulfur was found in continuously diminishing amounts in succeeding excretion periods. Inorganic sulfate and ethereal sulfate fractions of urine contained very small amounts of radioactivity. Feces contained 8 to 14% of administered radiosulfur, present primarily in neutral sulfur compounds. Table I contains data from typical experiments in which 100 γ of thiamine- S^{35} were administered to rabbits, and radiosulfur content of urinary and fecal sulfur fractions determined. Average recoveries of radiosulfur, obtained in urine fractions when the 3 dose levels were administered, are presented in Fig. 1.

In further experiments involving oral administration of thiamine- S^{35} , the oral dose of 100 γ of radiovitamin was followed 6 hours later by intramuscular injection of 2 mg of non-radioactive thiamine. This was done to determine if injection of the 20-fold excess of non-radioactive thiamine would cause increased excretion of radiosulfur from labeled vitamin. This increased excretion of radiosulfur, due to flushing out effect of excess non-radioactive thiamine injected was, in fact, realized, and an additional amount (average 17%) of administered radiosulfur was recovered. Practically all of this additional radiosulfur excreted was found in the neutral sulfur fraction of urine in the first 24-hour excretion period. Urinary and fecal recoveries of radiosulfur are shown in Fig. 2. For comparison, data are also presented from experiments in which 100 γ of thiamine- S^{35} were administered orally to rabbits without follow-up injection of non-radioactive thiamine, and urinary and fecal recoveries of radiosulfur determined for the same time. In contrast to the marked difference in excretion of radiosulfur in the neutral sulfur fraction of urine in these 2 series of experiments, inorganic sulfate and ethereal sulfate fractions of urine,

and fecal sulfur fractions show no significant differences in radiosulfur recovery.

In oral administration shown in Fig. 2, the main metabolites of thiamine-S³⁵ were isolated from urine collected in the first 24-hour excretion period. It was found that thiamine-S³⁵ accounted for approximately 60 to 65%, and thiazole-S³⁵ accounted for approximately 34% of neutral sulfur. These results are similar to those obtained by McCarthy *et al.*(1), and by Iacono and Johnson(6), who found that when rats were given intraperitoneal injection of thiazole-2-C¹⁴-thiamine, unchanged radiothiamine accounted for about 60% of radiocarbon of urine.

Parenteral administration. The radiovitamin was administered to rabbits by intramuscular injection, and by intravenous injection. In both series, thiamine-S³⁵ was dissolved in normal saline and pH of solution adjusted to 7.0. Injections of 100 γ were given in the musculature of hind leg, and in marginal ear vein, respectively. The results are presented in Fig. 3.

When radiovitamin was administered by intramuscular injection, average total recovery of radiosulfur in urine and feces was 86% for six 24-hour periods. Radiosulfur recovered in neutral sulfur accounted for 75% of administered dose. Average total recovery of radiosulfur, for the same time, when radiovitamin was administered by intravenous injection, was 55%, of which about 41% was in the neutral sulfur fraction of urine. For both parenteral routes, as with oral administration, inorganic and ethereal sulfate fractions of urine, and fecal sulfur fractions are minor excretory pathways for radiovitamin and its metabolites.

The results of our intramuscular injection experiments are similar to those obtained by other workers who studied the fate of intramuscularly injected thiamine-S³⁵ in other species. McCarthy and coworkers(1) obtained essentially the same results after intramuscular injection of 50 γ of thiamine-S³⁵ to rats. Borsook and his associates(7), though working with pharmacological doses of radiovitamin, obtained similar results with a human subject. Six days after the last of 4 daily in-

jections of thiamine-S³⁵ (16 mg), 61% of radiosulfur had been recovered in urine, and 11% in feces.

Studies on fate of intravenously injected radioactive thiamine have not been reported. As data in Fig. 3 indicate, recovery of radiosulfur is considerably lower than that obtained by either of the other routes used. A possible explanation is that, since radiovitamin is introduced directly into the bloodstream, it may be removed from the blood and stored by liver and other organs to a larger extent than when administered orally or intramuscularly, and is therefore retained and excreted over longer periods of time.

Summary. The fate of thiamine, labeled with sulfur-35 in the thiazole moiety, has been studied in rabbits after oral and parenteral administration of doses in the physiological range. Analyses of the urinary and fecal sulfur fractions for 4 to six 24-hour periods following administration of radiovitamin, gave average total recovery of radiosulfur of 77% after administration by stomach tube, 86% after intramuscular injection, and 54% after intravenous injection. When oral dose of radiovitamin was followed 6 hours later by intramuscular injection of a 20-fold larger dose of non-radioactive thiamine, an additional 17% of radiosulfur was recovered in urine. In all cases, the neutral sulfur fraction of urine contained more than 50% of the recovered radiosulfur, and the greater portion of this was excreted in the first 24-hour period following administration of radiovitamin. Isolation of main metabolites following oral administration indicates that unchanged thiamine-S³⁵ and thiazole S³⁵ moiety together account for approximately 95% of radiosulfur of the neutral sulfur fraction in the first 24-hour period, and are excreted in a ratio of 2 : 1, respectively.

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THIAMINE METABOLISM

WITH PARTICULAR REFERENCE TO THE ROLE OF THE
LIVER AND KIDNEYS

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Banga, Ochoa and Peters¹ have shown that all of the biologic activity of thiamine is exerted only when the latter exists in the form of diphosphothiamine. All of the nucleated cells of the body have been thought to possess the power to convert thiamine to diphosphothiamine,² the liver³ and the kidney⁴ possessing this activity more than the other tissues. Furthermore, these organs have a greater capacity for the dephosphorylation of diphosphothiamine.⁵

These observations introduce an important question to whether with severe disease of the liver or kidneys there is a disturbance in thiamine metabolism.⁶ Certain studies that have been made suggest that such may be the case. For example, Banga⁷ found that 3 patients with hepatic cirrhosis excreted a distinctly larger percentage of a standard test dose of thiamine than did his normal subjects. He suggested that the damage of the liver might cause a decrease in the phosphorylation of thiamine and lead to a decrease in the storage and utilization of this vitamin. A somewhat similar phenomenon was observed by Williams, Egana, Robinson, Asper and Dutoit⁸ in

thyrotoxic patients. They found that some of these patients excreted normal amounts of thiamine in the urine even when the diphosphothiamine content of the blood was subnormal. The frequent existence of impaired hepatic function in thyrotoxicosis and the resulting decrease in the phosphorylation capacity of the liver were suggested as one explanation for the inefficient thiamine economy.

Thiamine deficiency is often present in patients with hepatic cirrhosis. A history of a low thiamine intake is usually obtained, but this may not be the only factor involved in the reduction of storage of the vitamin. Since thiamine is used in the treatment of hepatic cirrhosis, it is of interest to know more about its utilization in these patients.

Less attention has been paid to the relationship of disease of the kidney to thiamine metabolism. However, it has been observed in patients with renal disease⁹ and with congestive heart failure⁸ that a subnormal quantity of a test dose of thiamine is excreted.

In an effort to elucidate further the role of the liver and the kidneys in thiamine storage, we have studied certain phases of thiamine metabolism in persons with severe disease of these organs.

PLAN OF STUDY

It was our belief that if the liver and the kidneys play an important role in the phosphorylation of thiamine it might be possible to demonstrate an impairment in this process in persons with severe disease of the liver or kidneys by administering a standard amount of thiamine and determining the blood levels of it frequently. Furthermore, the capacity for dephosphorylation perhaps can be estimated by administering a standard amount of diphosphothiamine and comparing its rate of breakdown with that in health.

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¹ From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard), Boston City Hospital, and the Department of Medicine, Harvard Medical School.

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All of the subjects with severe disease of the liver had Laennec's cirrhosis. They had had pronounced ascites, but at the time the test was performed little or no fluid was present. No patient was jaundiced at the time of the test. No more than a slight anemia was present in any subject, and there was no leukocytosis. Although several of these patients were probably deficient in thiamine at the time they were admitted to the hospital, each patient was given 15 mg. or more of thiamine hydrochloride per day for at least one week. In an effort to avoid having these patients supersaturated with thiamine at the time of the test, the supplementary thiamine was not given for at least three days preceding the test. Evidence of pronounced impairment of hepatic function was secured by means of the hippuric acid test and the bromsulphalein test. All patients had been hospitalized for several weeks preceding the test, and on the day of the test they were kept in bed in a fasting and resting state.

All of the subjects with severe disease of the kidneys had both chronic glomerulonephritis with pronounced azotemia. Many of these patients had more than a mild degree of anemias and leukemias. The same precautions for avoiding thiamine deficiency were taken as in the group with cirrhosis. There was little deviation of the blood from normal, except for moderate anemia in 1 patient.

The normal subjects were college students. On the day of the test the student came to the hospital without breakfast and with as little exertion as possible. He then lay down for thirty minutes before the test was begun and remained in a basal condition until the test was completed.

After a fasting specimen of blood was taken, 15 mg. of thiamine hydrochloride was injected intravenously during a period of exactly two minutes.¹⁰ Immediately after the injection a sample of blood was taken from the opposite arm. Samples, about 15 cc. each, were taken subsequently at six minutes, fifteen minutes and one hour. Twice this amount was obtained as the fasting specimen in order that a thiamine recovery experiment could be performed. Potassium oxalate was used as an anticoagulant, the final dilution being 0.2 per cent. Immediately after collection each sample of blood was placed in a pan of ice and kept there until the analysis for the thiamine and diphosphothiamine was begun, about fifteen to thirty minutes later.

The experiment was repeated with other groups of normal, cirrhotic and nephritic persons, 15 mg. of cocarboxylase¹¹ (diphosphothiamine) being used instead of thiamine. In this experiment the blood cells and the plasma were analysed individually for the content of thiamine and diphosphothiamine.

The normal subjects were studied also for changes in the thiamine and diphosphothiamine levels of the blood after the administration by mouth of 15 mg. of thiamine in about 3 ounces (90 cc.) of water.

Immediately on the completion of each test the subject was requested to empty his bladder. The urine was preserved with glacial acetic acid and analyzed for thiamine.

Methods of Thiamine and Diphosphothiamine Analysis.—The method employed for the estimation of thiamine

in the urine was described by Egana and Melckjohn.¹² This method is a modification of the ones used by Jansen,¹³ by Hennessey and Cerecedo¹⁴ and by Harris and Wang.¹⁵ It depends on the oxidative conversion of thiamine to thiochrome in the presence of alkaline ferricyanide, giving a bluish fluorescence under ultra-violet radiation, which can be quantitated by a comparison with a set of standards. This method has proved to be relatively accurate for the estimation of thiamine in the urine. The total twenty-four hour excretion of normal persons has been found to range from 35 to 250 micrograms.

In the estimation of the thiamine and the diphosphothiamine content of blood we have used the methods of Egana and Robinson.¹⁶ The technique is similar to that for the determination of thiamine used on urine. The content of diphosphothiamine in the blood is similar to that in the urine. It is estimated on the same specimen of blood as is used for the determination of thiamine. This is possible because diphosphothiamine is converted into diphosphothiochrome in the presence of alkaline ferricyanide and the diphosphothiochrome can be separated from thiochrome, owing to the insolubility of the former in isobutyl alcohol. The standards for diphosphothiochrome are prepared by running known amounts of diphosphothiamine through the same procedure as the unknown. The amount of blue fluorescence is compared with standards, as in the case of thiochrome, and thereby the amount of diphosphothiamine determined.

Great care must be taken to avoid any hemolysis, since it has been our experience as well as that of others¹⁷ that this leads to large errors in the estimations of thiamine and diphosphothiamine.

The thiochrome method is not as accurate when applied to blood as when applied to urine. In the former there are more interfering substances and the percentage of recovery of added thiamine is more variable. Similar difficulties are encountered with the determination of diphosphothiamine. However, these methods may be used for the estimation of pronounced relative changes such as we are presenting in this study.

RESULTS

(a) Results of Administration of Thiamine.

After the administration of 15 mg. of thiamine hydrochloride by mouth to the normal subjects a rapid absorption of this substance was evidenced by a distinct rise in the thiamine level

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10. Originally the test dose was 5 mg., but it was increased to 15 mg. because the former amount did not cause as much change in the thiamine level of the blood as we desired. We preferred not to use a larger dose than necessary.

11. The cocarboxylase was supplied by Merck & Co., Inc.

of the blood six minutes later (chart 1). The diphosphothiamine rose concomitantly and continued to increase for fifteen to thirty minutes; at the end of one hour it was still above normal.

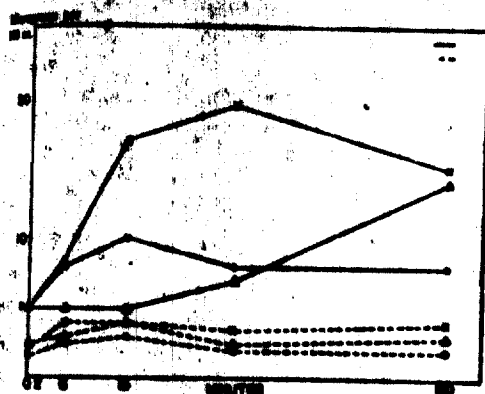


Chart 1.—The changes in the thiamine (dotted lines) and diphosphothiamine (solid lines) content of whole blood of 3 normal subjects following the oral ingestion of 15 mg. of thiamine hydrochloride.

The elevation of the thiamine level was of less degree than that of the diphosphothiamine, and it tended to return to normal more quickly. The amount of thiamine excreted in the urine was 0.6 per cent (range 0.3 to 0.8 per cent) of the injected dose.

When thiamine was administered intravenously to normal subjects the changes in the blood

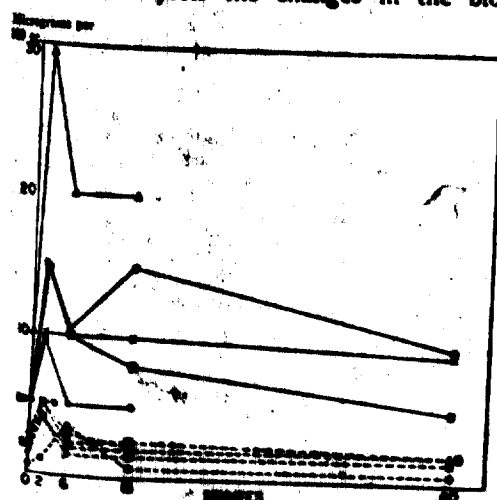


Chart 2.—The changes in the thiamine (dotted lines) and diphosphothiamine (solid lines) levels of the blood following the intravenous injection of 15 mg. of thiamine hydrochloride into 3 normal subjects. Each injection was given during a period of two minutes.

took place much more quickly (chart 2) than when it was given by mouth.¹⁰ The thiamine

is. The great variability in the responses of the normal subjects, as well as in those of the abnormal ones,

level of the blood reached a peak immediately on completion of the injection, but within six minutes it was normal. The rapid rate of phosphorylation was demonstrated by the large amount of diphosphothiamine found in the two minute sample. Thereafter the level declined, although in 2 instances it was still elevated at the end of an hour.

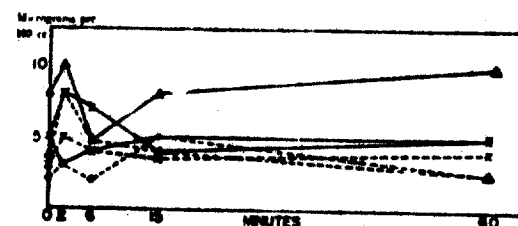


Chart 3.—The changes in the thiamine (dotted lines) and diphosphothiamine (solid lines) of the blood of 3 patients with hepatic cirrhosis given intravenously 15 mg. of thiamine hydrochloride.

In the patients with cirrhosis (chart 3) there was distinctly less rise in the diphosphothiamine content of the blood than occurred in the normal group (chart 2). This fact plus the slightly greater rise in the thiamine level of the patients with cirrhosis suggests that phosphorylation had taken place less readily, as discussed later.

In patients with nephritis (chart 4) the rise in the thiamine level developed more slowly than in the normal subjects, but the level eventually reached about the same height. This delay may have been due to an increase in the circulation

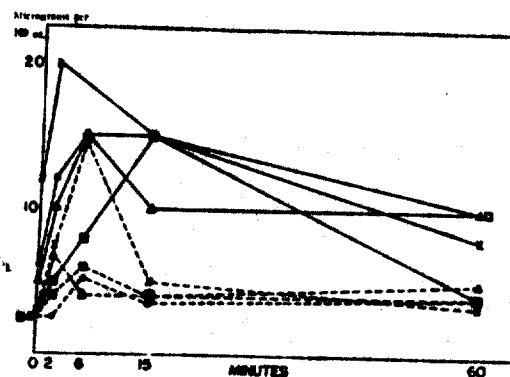


Chart 4.—The changes in the thiamine (dotted lines) and diphosphothiamine (solid lines) of the blood of 4 patients with chronic nephritis given 15 mg. of thiamine hydrochloride intravenously.

time. The amount of diphosphothiamine formed is essentially the same as in the normal group.

Although in the three groups of subjects a striking increase in the diphosphothiamine content of the blood occurred after the injection of

is shown by the curves in the figures. Therefore, in our interpretations we have considered the trends rather than the specific curves.

thiamine, this rise was not of sufficient magnitude to account for more than 5 per cent of the amount injected. This leads to the questions as to (1) whether most of the thiamine was excreted in the urine, (2) whether it was stored in the tissues or (3) whether it was broken down to substances which do not give the thiochrome reaction.

During the hour that these tests were conducted the normal subjects excreted in the urine, on the average, 0.5 per cent of the oral dose of thiamine and 2.5 per cent of the intravenous dose. The patients with cirrhosis on the average excreted 4.7 per cent, and those with nephritis 1.4 per cent. Tabor¹⁹ stated that the majority of the excretions of thiamine B₁ and its metabolites in the urine of normal subjects in the first 24 hours after administration of the vitamin would not account for more than 10 per cent of the administered thiamine. Furthermore, if the pyridoline accelerator of yeast fermentation²⁰ were also included, this would not account for much more of the vitamin. As to how much of the thiamine was broken down during the period of observation, the present data are inadequate for a good estimation.

The chief consideration is storage of thiamine—its rate of storage, site and extent. The work of Borsook and associates²¹ is of interest in this connection. They injected intramuscularly thiamine containing radioactive sulfur and determined the amount of the latter excreted in the urine and feces. One normal subject was given 16 mg. daily for four days. On the first day less than 20 per cent of the injected thiamine was excreted but many times the usual amount of nonradioactive thiamine was eliminated. Six days after the last injection a total of 61 per cent of the injected radio-sulfur had been recovered from the urine and 11 per cent from the feces, but 28 per cent remained unaccounted for.

Ferrebee, Weissman and Owen²² found that the total amount of thiamine in the body is about

25 mg., the heart containing about 2 to 3 micrograms per gram, the skeletal muscle 0.5 microgram and the liver, kidney and brain about 1 microgram each. These investigators found that whereas a striking increase in the thiamine content of the tissues resulted from the feeding of large doses of this vitamin to rats and to human beings who were deficient in this substance, only slight increases (about 10 to 20 per cent) occurred in men maintained on a normal diet. However, it is significant that only a slight increase in the thiamine content of the tissues could cause an appreciable reduction in the thiamine content of the blood, since the normal level of the latter is about 7 micrograms per hundred centimeters, which is about one-fiftieth that of the tissues.

Mason and Williams²³ adduced evidence that an appreciable amount of storage of thiamine may result in man. They fed 2 normal subjects 7.5 mg. of thiamine hydrochloride for thirty-seven days and then placed them for five days on a diet containing 400 micrograms of thiamine and for twenty-two days on a diet which furnished 600 micrograms. Thereafter, the daily twenty-four hour excretion of thiamine was normal, as was the response to a test dose of thiamine.

Leong²⁴ found that in rats maintained on a normal stock diet the concentrations of thiamine in the various tissues was essentially the same. However, with an excessive ingestion of this vitamin the liver and heart showed a distinct increase in storage while the other organs showed little change.

Ochoa and Peters²⁵ showed that in rats and pigeons' tissues almost all of the thiamine present was in the form of cocarboxylase. This is of interest, since Banga, Ochoa and Peters² showed that essentially all of the activity of thiamine in the oxidative decarboxylation in tissue was proportional to the amount of cocarboxylase present. Furthermore, Ochoa and Peters demonstrated that the amount of diphosphothiamine was much reduced in pigeons with B₁ avitaminosis.

Ochoa and Peters found that thirty minutes after injection of vitamin B₁ into avitaminous pigeons and rats there was a pronounced accumulation of cocarboxylase in the liver, whereas

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little or no increase developed in the skeletal muscle, heart or brain. The level of cocarboxylase in the liver could not be made to rise much above normal. Normal animals given thiamine had only a slight rise in the cocarboxylase of the tissues. Ochoa⁸ obtained somewhat similar results for the *in vitro* synthesis of cocarboxylase from thiamine, using liver, brain, intestine and breast muscle of pigeons. Of these tissues the liver was the only one which exhibited a good capacity to phosphorylate added thiamine to diphosphothiamine. He found that the amount of synthesis soon reached a maximum, which was generally slightly above the normal concentration of cocarboxylase, beyond which no greater concentration of diphosphothiamine resulted, no matter how much thiamine was added. The maximal synthesis in the liver resulted in thirty to sixty minutes, but a short time thereafter the level began to drop. Brain and muscle were found to show less synthesis, but there was no decrease in the amount of cocarboxylase after the maximum was reached. Westmabrink and Goudamit⁹ showed that the kidneys were active in the phosphorylation of thiamine. Goodhart and Sinclair¹⁰ found no evidence of the synthesis of cocarboxylase from thiamine in normal pigeon, ox and human bloods. However, when this vitamin was added to thiamine-deficient pigeon blood, marked synthesis resulted. These investigators also reported that only about 14 per cent of the total thiamine of the blood is in the plasma and essentially all of this is in the free form. On the other hand, almost all of this vitamin in the blood cells is in the form of diphosphothiamine, the white cells containing many times the amount in the red cells.

In summary, the data accumulated thus far suggest that most of the thiamine of the body is phosphorylated before it is stored, the liver and kidneys being particularly active in phosphorylation. The storage can be increased above normal, but there is a limit, which is not much above normal, beyond which no appreciable increase in the stores results. Presumably all tissues share in this accumulation, although not to an equal extent. Immediately after large quantities of thiamine have been injected the liver takes the chief responsibility for the storage and dispensation of this vitamin, permitting its concentration to increase greatly for a temporary period, during which time an establishment of equilibrium is taking place. In this interval some of the diphosphothiamine of the liver is dephosphorylated, the thiamine, thereby liberated being carried by the plasma to other tissues, where it is again phosphorylated. It may also be transported in the blood cells, chiefly in the form of diphospho-

thiamine. An excess of vitamin to that stored in the body is broken down or excreted as thiamine in the urine. As the body stores go below normal, the phosphorylating activity of the kidneys increases and thereby aids in the economy of thiamine by preventing its excretion in the urine.

In attempting to interpret the various changes in the dynamic equilibrium of thiamine on the basis of studies of the blood and urine, certain facts should be emphasized. It has not been established how satisfactory the thiamine level of the blood is as an index of the content of the vitamin in the tissues. The total amount of thiamine in the blood is small, and its concentration in the tissues of the body varies. Furthermore, it is known that when large doses of the vitamin are injected the amount taken out by the tissues varies a great deal.²⁰

The fact that in the cirrhotic patients as compared with normal there are less of a rise in the diphosphothiamine, a greater rise in the thiamine and an increased excretion of thiamine in the urine suggests that there is a decrease in the phosphorylation process. If all of the diphosphothiamine of the blood is a result of the phosphorylating activity of the blood cells, it is probable that the liver has an indirect effect on this process (charts 2 and 3). It is possible that the blood cells may receive diphosphothiamine as such from the liver cells, during their passage through the liver. If this hypothesis is correct, in subjects with cirrhosis one would expect a lower level of diphosphothiamine of the blood than one observes in normal subjects, since in the former group there is a decreased blood flow through the liver and a decrease in most of the activities of the liver cells. The *in vitro* studies of Banga, Ochoa, and Peters¹ with various preparations of brain tissue suggested that cocarboxylase permeates the cell wall not as such but in the form of thiamine, which after entering the cell is phosphorylated. Although it is probable that the phosphorylating action of the kidney is impaired with severe renal disease, there are no definite manifestations of this reflected in the blood.

(b) *Results of Administration of Diphosphothiamine.*—After the intravenous administration of 15 mg. of cocarboxylase to normal subjects the plasma level rose rapidly (chart 5) and in 1 subject reached 100 micrograms per hundred centimeters. However, the rapid rate of dephosphorylation is demonstrated by the increase in the thiamine level in the two minute specimen. The thiamine content of the plasma tended to return to normal in six to fifteen minutes, but the diphosphothiamine level remained elevated for more than an hour.

Concomitant with the changes in the plasma, rapid changes occurred in the blood cells (chart 6). A marked increase in the diphosphothiamine was observed in the two minute specimen. There was also a slight increase in the thiamine content

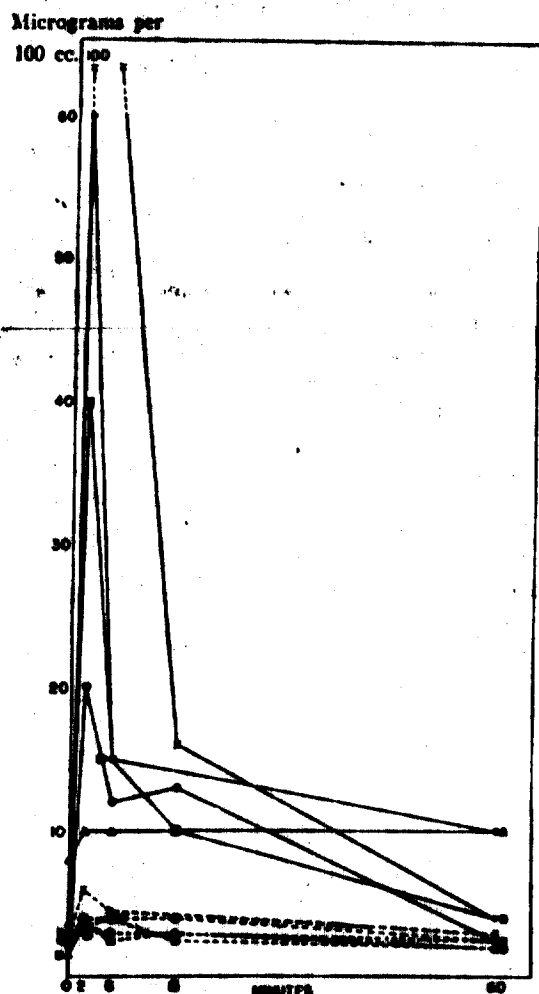


Chart 5.—The changes in the thiamine (dotted lines) and diphosphothiamine (solid lines) content of normal blood following the intravenous injection of 15 mg. of coenzyme in 4 subjects. The injections were given during a period of two minutes.

of the cells. However, since the increase in the thiamine content of the cells and plasma was relatively small as compared with the increases in the diphosphothiamine content, one is prompted to consider either that diphosphothiamine passes through the cell membrane as such or that it is dephosphorylated and then rephosphorylated at an extremely rapid rate. The latter consideration introduces the query as to how great a part the blood cells alone play in regulating the interconversion of phosphorylated thiamine and free thiamine. Goodhart and Sinclair² could not

demonstrate in vitro any synthesis of diphosphothiamine from thiamine in normal blood.

Since the liver and kidneys have been demonstrated to be active in dephosphorylating as well as in phosphorylating thiamine, it is important to compare the observations on the normal subjects with those on persons with hepatic cirrhosis and severe nephritis.

In the cirrhotic group (chart 7) there was a distinctly smaller rise in the diphosphothiamine content of the plasma than occurred in the normal group. Furthermore, in the former group the diphosphothiamine had attained normal levels within six minutes, whereas with the latter group it was still elevated at the end of an hour. The diphosphothiamine content of the blood cells also tended to increase somewhat less in the cirrhotic

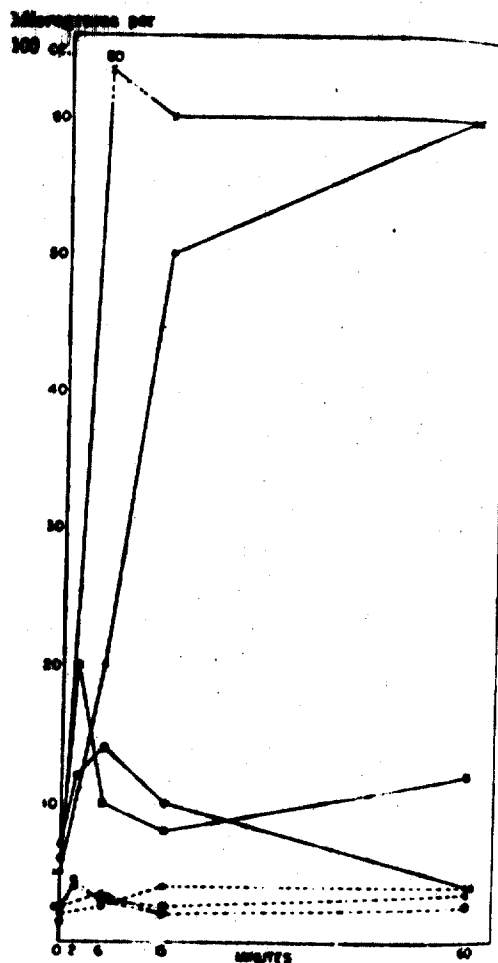


Chart 6.—Same subjects as in chart 5. The curves show the changes in the thiamine (dotted lines) and diphosphothiamine (solid lines) content of the blood cells.

group (chart 8). On the other hand, the plasma thiamine of the cirrhotic group rose more than did that of the normal group. The changes in

in cellular thiamine were similar in the two groups.

The alterations in the thiamine and diphosphothiamine blood levels in the nephritic patients (charts 9 and 10) were somewhat intermediate those of the normal and the cirrhotic subjects.

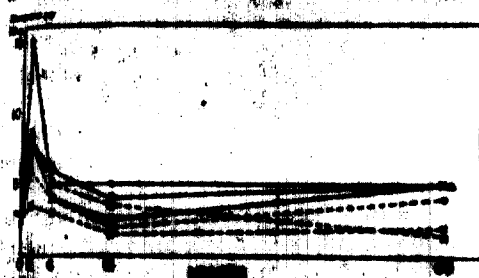


Chart 7.—The changes in the thiamine (dotted lines) and diphosphothiamine (solid lines) content of the plasma of 3 patients with nephritis after the intravenous injection of 15 mg. of cocarboxylase. Compare with chart 5.

During the hour that the test was conducted the amount of thiamine excreted in the urine by the normal subjects was 1.4 per cent of the injected dose of diphosphothiamine; the nephritic group excreted 3.4 per cent. Specimens of urine were obtained from only 1 of the cirrhotic patients. However, he excreted 45 per cent of the injected dose of diphosphothiamine. The urinary thiamine was in the free form and therefore not phosphorylated. In investigating the cause of the striking diuresis of thiamine, it was discovered that one hour before the diphosphothiamine

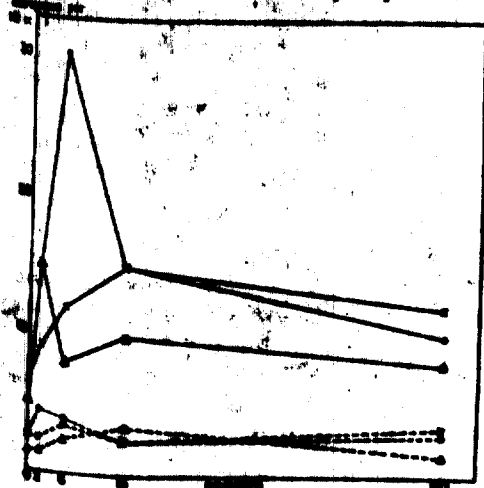


Chart 8.—The changes in the thiamine (dotted lines) and diphosphothiamine (solid lines) content of the blood cells. Subjects same as in chart 7.

test was made the patient had been given 2 cc. of mercurphylline. Because of this, further observations were made on the effect of mercurphylline on excretion of thiamine.

Effect of Mercurphylline on the Excretion of Thiamine in the Urine.—We first studied the effect of mercurphylline on the excretion of thiamine in the urine of 2 normal subjects. We then studied its effect on 2 patients with slight congestive heart failure and 1 with Laennec's cirrhosis, since these types of patients are the ones which most often are treated with mercurphylline.

The effect of mercurphylline on excretion of thiamine was observed when it was given in conjunction with 15 mg. of thiamine hydrochloride. These two drugs are frequently given together; hence such a study seemed appropriate.

All of the mercurphylline²⁵ was administered intravenously in 2 cc. amounts two hours before

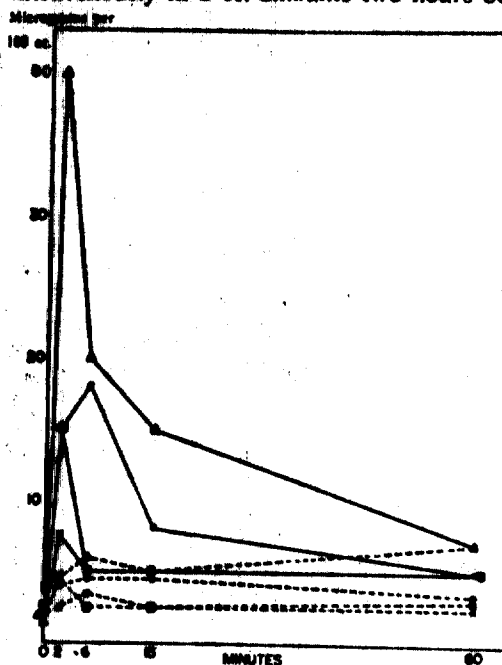


Chart 9.—The changes in the thiamine (dotted lines) and diphosphothiamine (solid lines) content of the plasma of 4 patients with chronic nephritis given 15 mg. of cocarboxylase intravenously.

thiamine hydrochloride was administered. The latter was injected intravenously in quantities of 15 mg. into all the patients with the exception of patient N. M., who received doses of 1 mg. The urine was collected in glacial acetic acid for twenty-four hours following the injection of thiamine. The same dosage of these drugs was used on the days when they were given singly as when given together.

Normal subject A. M., having fully recovered from pneumonia, was transferred to the metab-

²⁵ The mercurphylline was tested for fluorescence but was found to have none. Therefore, this drug caused no apparent interference with the estimation of thiamine in the urine.

olism ward. Here he was maintained on the regular hospital diet with the addition of 6 tablets of brewers' yeast daily. After one week on such a regimen, estimates were made of the daily excretion of thiamine in the urine. On the fifth day thiamine was given intravenously. In the succeeding twenty-four hours 1.9 mg. of thiamine was excreted (chart 11). On the thirteenth day the subject was given mercurophylline, and during the succeeding twenty-four hours 10 mg. of thiamine was excreted.

Subject S. M., convalescent from an operation for an ingrown toe nail, was transferred to the metabolism ward and was prepared with a dietary regimen, as in the case of A. M. He was given thiamine hydrochloride alone, mercurophylline alone and then the two together. The mercurophylline alone had no definite diuretic effect on the excretion of thiamine. However, when

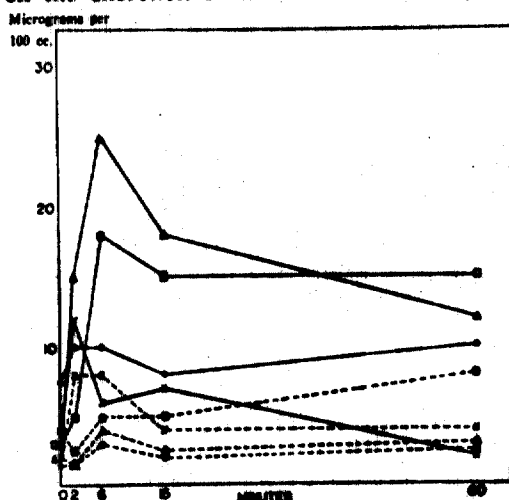


Chart 10.—The changes in the thiamine (dotted lines) and diphosphothiamine (solid lines) content of the blood cells. Subjects same as in chart 9.

thiamine and mercurophylline were given on the same day there was a distinctly greater excretion of thiamine (1.06 mg.) than when the latter was given alone (0.38 mg.).

Patient W. E., a woman aged 70, had been in the hospital for several weeks with congestive heart failure due to arteriosclerotic heart disease. When we studied her, there was only a slight amount of heart failure. She had received subcutaneously 10 mg. of thiamine hydrochloride for several weeks. The injections were discontinued one week before our studies were begun. A greater diuresis of thiamine resulted when mercurophylline was given on the same day as the thiamine (0.26 mg.) than occurred when the latter was given alone (0.14 mg.). However, when mercurophylline alone was given, the most pronounced diuresis of thiamine resulted (12.6 mg.).

Patient M. U., a woman aged 50, had been in the hospital for several weeks with congestive heart failure because of chronic rheumatic heart disease. Her dietary intake preceding hospitalization was essentially normal, and she was maintained on a "cardiac" diet, without vitamin supplements, while in the hospital. At the time that our studies were conducted only slight manifestations of congestive failure remained. Mercurophylline given in conjunction with thiamine hydrochloride did not increase the diuresis of thiamine. However, when mercupurin alone was given, a pronounced diuresis of thiamine resulted (6.6 mg.).

The patient with cirrhosis (N. M.) was a man aged 35 who had been in the hospital for several weeks. His diet had been supplemented with several vitamins, including thiamine. However, he had received no extra vitamins during the week preceding our studies. When mercurophylline was used alone, there was no definite increase in the amount of thiamine excreted. However, when it was used in conjunction with thiamine, more of this vitamin appeared in the urine (0.28 mg.) than was found after the administration of thiamine alone (0.14 mg.).

Therefore, it is apparent that different subjects excrete variable amounts of thiamine in the urine after the administration of mercurophylline; sometimes this amount is enormous. This increased elimination of thiamine is not due alone to an increase in the volume of urine, since it was found that in several instances there was an increase in the concentration of this vitamin in the urine (chart 11). Indeed, in the urine of 2 subjects the concentration was about fifty times greater than normal, although no extra thiamine had been given for more than ten days. In spite of the excretion of large quantities of thiamine in the urine, it contained no demonstrable diphosphothiamine.

The observations which have been made thus far suggest that the large capacity of the kidney for the interconversion of thiamine and diphosphothiamine is for the conservation of the body's supply of thiamine, the kidney tubules reabsorbing most of the thiamine when its cells are deficient in this vitamin and converting it into diphosphothiamine, which, in turn, can liberate thiamine for dispensation by the blood. On the other hand, when the body is saturated with the vitamin, the kidneys permit its escape in excess quantities. When mercurophylline is administered, the tubular activity of the kidneys can be so disturbed as to permit the elimination of rather large quantities of thiamine in the urine. We found this response to vary a great deal in different subjects.

SUMMARY AND CONCLUSIONS

Thiamine hydrochloride administered to normal subjects by mouth was found to be rapidly absorbed from the gastrointestinal tract and converted into diphosphothiamine within a few hours.

When 15 mg. of thiamine hydrochloride was administered intravenously to normal subjects, an immediate and striking increase in the diphosphothiamine level of the blood was observed. The rapid

When 15 mg. of cocarboxylase was administered intravenously to normal subjects, an increase in the diphosphothiamine and the free thiamine of the plasma and the blood cells was observed immediately. The thiamine level rapidly returned to normal, but the diphosphothiamine remained elevated, for more than an hour in some instances. In patients with advanced cirrhosis there was also an immediate increase in the free thiamine level of the blood, but there

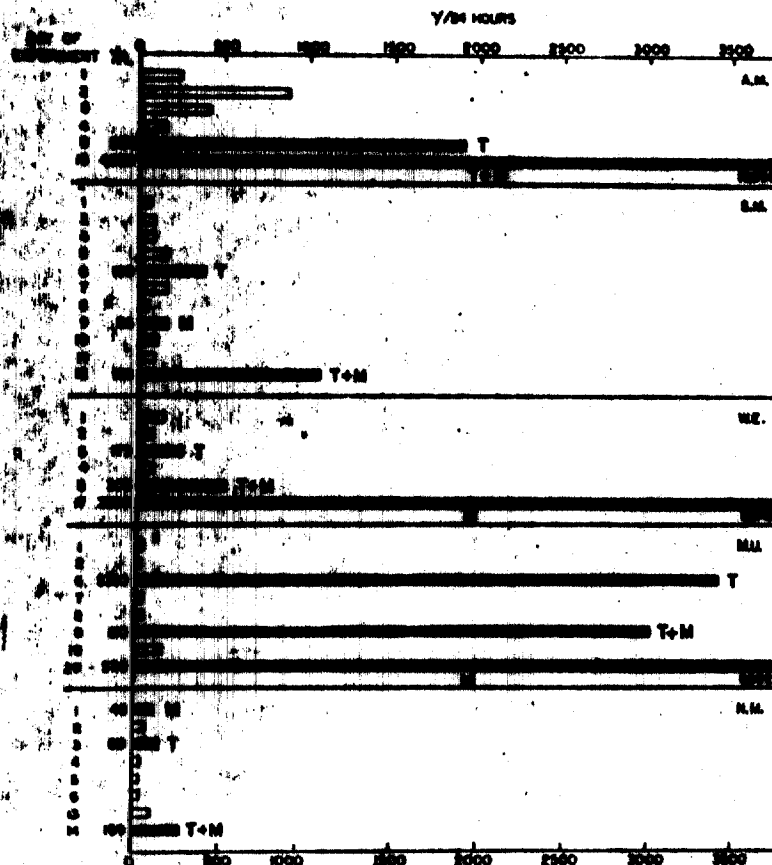


Chart 11.—The changes in the twenty-four hour urinary excretion (in micrograms) of thiamine (T) following the intravenous injection of 2 cc. of mercuraphylline (M). When thiamine and mercuraphylline were both given, the former was given immediately two hours after the latter. The amount of thiamine hydrochloride injected was 15 mg. for each patient except W. E. who received only 1 mg. Note that mercuraphylline caused a marked increase in the excretion of thiamine. This increased excretion was not due to just an increased volume of urine. (Values at the left of the solid bars represent micrograms per liter.)

disappearance of the vitamin from the blood was shown by the fact that the thiamine level of the blood was normal within six minutes and the fact that the total diphosphothiamine of the blood never represented more than about 5 per cent of the injected dose.

In patients with severe hepatic cirrhosis, an impairment in the phosphorylation of the thiamine was demonstrated, but not much disturbance in the excretion was observed in patients with nephritis.

was much less rise in the diphosphothiamine than occurred in the normal subjects. In patients with severe nephritis, the changes were intermediate between those of the normal and of the cirrhotic groups.

Mercuraphylline was found to cause the excretion of a large amount of thiamine. This effect was not due alone to an increased elimination of water, because in some instances the concentration of the thiamine in the urine was many times that found in normal urine.

**TOXICOLOGICAL AND BIOCHEMICAL STUDY OF TWO VITAMIN B₁
DERIVATIVES WITH HIGHER RESORPTION THAN THIAMINE WHEN
ORALLY ADMINISTERED**

**NOTE 1. BLOOD CONTENT LEVELS IN RATS AND LD₅₀ VALUES
OF DERIVATIVES ADMINISTERED P.O. AND I.V. TO MICE .**

by

**D. Winter, Ingeborg Hadrich, Sanda Sanvare
and Cornelia Stancescu**

612.2

Summary. - The median lethal doses (LD₅₀) of two thiamine derivatives with open thiazole ring, namely the dithio-propyl-thiamine (D.T.P.) and the S-ethyl-octanoyl-thiamine (S.E.C.T.) were determined comparatively to thiamine hydrochloride. The ratio between the values of LD₅₀ when the derivatives are orally and intravenously administered is 1/8 and respectively 1/12, whilst it is 1/90 for vitamin B₁, which proves that comparatively to vitamin B₁, their resorption is higher, when they are orally administered.

Following p.o. administration of equimolecular doses of the three substances, the values found for the blood content levels were 20-50 times greater in the case of the derivatives than for vitamin B₁. Even when 10 times smaller doses of both derivatives were used, between 15 minutes and 24 hours, the blood content levels were higher and persisted longer than when vitamin B₁ was administered.

Parenteral administration of vitamin B₁ brings rapidly about the efficient concentration in blood and tissues, while oral administration requires for the same results

very high doses due to the deficient resorption of the vitamin at the intestine level and never attains the concentrations obtained in case of parenteral administration.

This deficient resorption is due on one hand to the physical and chemical properties of the vitamin, (low liposolubility and decomposition in presence of thiaminase), and on the other hand, to the particularities of its metabolism, as it is partially resorbed as such, and partially under the action of the intestinal flora.

Organisms have a resorption threshold for the phosphorylated form (T. P. F., thiamine-pyrophosphate), and never form vitamin B₁ deposits, because the excess amounts are continuously eliminated (3), (12), (14).

For oral administration of vitamin B₁ in large doses, showed that above a certain dose, resorption reaches its utmost value, which in some cases will prevent it to attain the efficient therapeutical concentrations required (10). Moreover, the ratio between parenteral and oral resorption is indicated by the very low value of the ratio between the i.v. LD50 and the p.o. LD50, which for some animals is of approximately 1/40 (12).

In 1952, Fujiwara and Watanabe (5) isolated for the first time an open thiazole ring thiamine (allil-thiamine), following which many other thiamine derivatives appeared in the literature, all of which having a p.o. resorption higher than vitamin B₁. From then on, it became possible to obtain also oral vitamin B₁ efficient medications.

The chemical properties common to the thiamine derivatives with an open thiazole ring, refer to : their capability to give definitely crystallized and stable bases ; their high liposolubility ; the lack of thiochrome direct reaction as in the vitamin B₁ specific reaction (with closed thiazole ring) (6).

From the pharmacological and biochemical point of view, these derivatives have a few common characteristics, namely: they change into vitamin B₁ when introduced in organisms (are recycled in the liver, due to the presence of reducing agents as cystein or glutathione, or to an enzymatic intervention (6), raising the level of the hepatic cecarboxylase (6-8)); their resorption is higher than that of vitamin B₁ when orally administered (1), (6), (9), (10), (15), (16) ; their efficacy from the vitaminic point of view (experimental and clinical) compares to that of vitamin B₁.

In the present work, we give the results of determinations referring to the LD50 for mice and the resorption in case of

eral administration to rats, of the 2 vitamin B₁ derivatives with open thiazole ring, namely the dithio-propyl-thiamine (D.T.P) and the S-ethoxy-carbonyl-thiamine (S.E.C.T).

MATERIAL AND METHODS

1. The acute toxicity (LD50) of the products in case of oral and intravenous administration to mice was determined. Calculation of LD50 and of the safety limits was achieved by the graphic probit method (2). Administration frequency was low (1ml/100 g) in the case of intravenous administration. For a dose, lots of 10 male mice each, weighing 18-22 g, were used.

2. The blood content levels were checked in male rats, weighing 120-160 g each, after they had been orally administered the vitamin and its derivatives, in various doses and at different intervals of time.

At regular intervals of time after administration (45 and 90 minutes ; 3, 6, 12 and 24 hours), the animals were killed and blood was collected from their carotid artery. The blood samples were collected on sodium oxalate. The total vitamin B₁ content of these samples was determined by the Friedman method (4), (11), and in some of the cases the differential content of free and bonded vitamin was determined. In biological material, thiamine and T.P.F. are usually bonded to the proteins. By extraction in warm acid media, this bond is hydrolyzed, then thiamine is oxidized in alkaline media, and the fluorescence of the thiochrome obtained is read in u.v. The free and the bonded vitamin (T.P.F.) were dosed by determining the thiochrome before and after the hydrolysis of the extracts with phosphatase (the phosphatase was prepared by Westenberg's method (17) from Brewer's yeast).

Lots of at least 5 animals were used for each dose and interval of time. The following substances were used: thiamine hydrochloride, dithio-propyl-thiamine (D.T.P.) and S-ethoxy-carbonyl-thiamine (S.E.C.T.), which had been synthesized by a group of scientists of I.C.C.F. (Pharmaceutical and Chemical Research Institute), under the leadership of Eng. Mariana Ionescu and Eng. Eva Dragoi. The last two substances mentioned above, were administered to the animals under the form of extemporaneously prepared hydrochloride. The doses in which these substances were used are given in the following tables.

RESULTS

1. Acute toxicity for mice

The values of LD50 for various administration paths as well as the ratio between i.v. LD 50 and p.e. LD50 for the two products studied, are given in Table 1 :

Table 1
Median lethal doses of thiamine derivatives with open thiazole ring

a. Substance	b. LD ₅₀ (mg/kg i.v.)	c. Limita de securitate	d. LD ₅₀ (mg/kg p.o.)	e. Limita de securitate	Raportul	
					LD ₅₀ i.v.	LD ₅₀ p.o.
e. Clonidine B ₁	252	24,2-118	2224	6310-10000	1/190	
D.T.P. (derivative)	300	270-300	2642	1670-3649	1/8	
S.E.C.T. (derivative)	300	200-370	3200	2010-6007	1/12	

Key: a)= substance, b)= safety margin, c)= ratio, d) =LD50, e)= hydrochloride, f)= comma to be replaced by period.

These data indicate that :

- The derivatives D.T.P. and S.E.C.T. have a lower toxicity (statistically significant) than B₁, when intravenously administered, and a higher toxicity (statistically significant) when orally administered.

- The ratio between i.v. LD50 and p.o. LD50 thus indicates that the p.o. resorption of these derivatives is higher than that of vitamin B₁.

- No statistically significant differences are to be observed between the LD50 of D.T.P. and of S.E.C.T., but, D.T.P. appears to be more toxic than S.E.C.T., both when intravenously and orally administered.

2. Blood content levels in rats after p.o. administration

The results obtained 3 hours after a single dose of 500 mg/kg of vitamin B₁ (and equimolecular doses of both derivatives) was administered, are given in table 2. We see in this table, that the level obtained with the B₁ derivatives after 3 hours is 20-50 times higher than the level obtained with vitamin B₁, when equal doses are used.

The highest levels are obtained with S.E.C.T. (the differences between D.T.P. and S.E.C.T. are statistically significant).

Table 2

Levels of blood content 3 hours after p.o. administration of a single 500 mg/kg dose of thiamine hydrochloride and of equimolecular doses of both derivatives

a Substance	b Dose (mg/kg)	c Conc. µg/ml stage thiamine total	E.S.	d Rapport concentration derivative/B ₁
B ₁	500	5,7	±1,2	—
D.T.P.	330	127,0	±47	22
S.E.C.T.	330	207,0	±31	32

Key : a)= substance, b)= dose, c)= content µg/ml blood of total thiamine, d)= ratio of derivatives concentration/B₁, e)= commas to be replaced by dots.

Comparatively following the thiamine curve at various time intervals (45 minutes - 24 hours) due to the great difference between the concentrations obtained on one hand with B₁, and on the other hand with the 2 derivatives, differences which produce perturbations in the analysis method, we used 10 times smaller doses of the respective derivatives. The values obtained under these conditions are given in table 3. Even when 10 times smaller doses of the vitamin B₁ derivatives were used, the blood content level, at all the intervals of time between 45 minutes and 24 hours, is higher than the one obtained with vitamin B₁. The differences between S.E.C.T. and B₁ are statistically significant at all the time intervals and in the case of D.T.P. and B₁, at 12 and 24 hours.

Table 3

Blood content levels at various intervals of time after administration of a single oral dose of vitamin B₁ and of derivatives (concentration µg/ml, total thiamine in blood ± E.S.)

a Time de la administration	Vitamin B ₁ (300 mg/kg)	D.T.P. (33 mg/kg)	S.E.C.T. (33 mg/kg)
45 min ✓	2,1 ± 0,1	3,08 ± 0,6	31,0 ± 2,6
90 min	1,9 ± 0,2	—	—
3 hrs c	4,3 ± 0,7	5,75 ± 3,0	30,0 ± 8,2
11 2 hrs	3,8 ± 0,2	—	—
6 hrs	3,6 ± 0,4	—	—
12 hrs	6,9 ± 0,03	5,4 ± 2,3	8,0 ± 4,6
24 hrs c	0,7 ± 0,02	1,4 ± 0,1	2,1 ± 0,8

Key: a)= time after administration, b)= minutes, c)= hours, d)= commas to be replaced by periods.

In case of administration of S.E.C.T. the levels of the blood content are higher at all intervals, than those obtained when D.T.P. is administered, and after 24 hours the blood still contains a noticeable quantity of the vitamin, whilst after the same period of time, the quantities present, of vitamin B₁ or of D.T.P. are under 1 μ/ml.

For all the substances, the blood curve is similar and presents a maximum around a 3 hour interval. In order to study the esterification capacity of the derivatives in comparison with vitamin B₁, a number of tests and a parallel, free thiamine and T.P.F. dosage has been effected, 3 hours after the administration of the 500 mg/kg vitamin B₁ and of 55 mg/kg S.E.C.T. and D.T.P. Results are given in table 4.

Table 4

Blood content levels 3 hours after p.o. administration of a single dose of vitamin B₁ and derivatives (conc. μg/ml blood)

a. Substance	b. Dose (mg/kg)	c. Thiamine libera	d. Thiamine legata	e. Thiamine totală	f. % thiamine legata
B ₁	500 500	0,1 0,1	2,4 1,4	2,5 1,5	100 93
D.T.P.	55 55	0,8 1,9	7,7 5,1	8,5 7,0	99 72
S.E.C.T.	55 55	17 19	32 30	49 49	65 51

Key : a) = substance, b) = dose, c) = free thiamine, d) = bonded thiamine, e) = total thiamine, f) = bonded thiamine %
g) = commas to be replaced by periods.

These results lower slightly the big difference between the efficient blood concentrations of vitamin B₁ and of its derivatives, especially in the case of S.E.C.T. as it is known that T.P.F. - the bonded form - represents the active part of the vitamin. But even under these conditions, the superiority of the derivatives studied is maintained, as compared to vitamin B₁.

DISCUSSION OF RESULTS

Many research studies can be found in literature which show the superiority of the derivative (D.T.P.), when orally administered, as compared to vitamin B₁ (6-9); as regards the derivative S.E.C.T., substance which has been initially synthesized by Japanese authors, there are not many pharmaceutical researches to be found (15). Also,

there are no comparative studies to be found in literature regarding the toxicity or resorption of these two derivatives, indicating the superiority of one or the other of these two, in view of their utilization in therapeutics.

The pharmacological studies presented, enabled us to confirm in the first place the p.e. intense resorption of these two derivatives, in comparison to vitamin B₁, as shown by the high blood content level as well as by the advantageous ratio between the i.v and p.e. median lethal doses.

The values found during the toxicity determinations for vitamin B₁ and D.T.P. median lethal doses (LD 50), correspond to those in the literature (13), (17); also, these we found for the blood total thiamine concentrations are close to the values given by Niese and colab. (7), (8). As regards S.E.C.T., the data in literature refer only to human and rabbit blood content levels, which as found also by the Japanese authors, have much higher values than those obtained after administration of equal doses of vitamin B₁ (15).

Considering the ratio between the blood concentrations found when administering to rats S.E.C.T. and D.T.P., the superiority of S.E.C.T. is evident and we can see that it is possible to obtain with this substance, about 6 times higher blood content levels than in the case of D.T.P. Nevertheless, we must consider the possibility of some differences as regards the p.e. resorption, metabolism, esterification possibility etc. of these derivatives for different species of animals, and from this point of view, the results obtained with mice (in which case the p.e. toxicity of S.E.C.T. is lower than that of D.T.P.), indicate that the resorption of S.E.C.T. is lower for mice than for rats.

Moreover, we cannot directly transpose, from animals to man, the S.E.C.T. superiority over D.T.P., because even in the case of rats there is a difference between these two derivatives as regards their esterification degree, D.T.P. being esterified (bonded) in a higher degree than S.E.C.T.). Further research will solve these problems and enable to find out the therapeutical value of these two derivatives in view of their utilization in clinics.

CONCLUSIONS

1. The vitamin B₁ derivatives, with open thiazole ring, the D.T.P. and S.E.C.T. have a higher median lethal dose than vitamin B₁ when intravenously administered to mice but a lower LD 50, when orally administered.

2. When orally administered to rats, D.T.P. as well as S.E.C.T. determine higher and more persistent concentration

levels than vitamin B₁ .

3. The quantitative differences between D.T.P. and S.E.C.T. which appeared in the research studies made on animals, are : the toxicity of S.E.C.T. when orally administered is lower than the toxicity of D.T.P. when administered to mice in the same conditions ; the blood concentration levels are higher at all time intervals between 45 minutes and 24 hours, after S.E.C.T. oral administration than after D.T.P. administration to rats ; 3 hours after administration, the amount present of bonded S.E.C.T. is lower than it is when D.T.P. has been administered.

4. The thiamine derivatives with open thiazole ring, namely the D.T.P. and the S.E.C.T. have the same efficacy as vitamin B₁ , but their resorption in the organisms is higher when they are orally administered ; they appear to be substances with bright prospects for the future, as they will be used as medication for human beings instead of vitamin B₁ , being easy to take (p.o.), and representing an efficient administration of this vitamin, ; also as a biostimulant for bee-veterinary utilization, in which scope high concentrations of vitamins will be incorporated in feed (fodder).

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Chemical-Pharmaceutical
Research Institute of
Bucharest

STUDIUL TOXICOLOGIC ȘI BIOCHIMIC A DOI DERIVAȚI DE VITAMINĂ B₁ CU RESORBȚIE PER ORALĂ SUPERIOARĂ TIAMINEI

NOTA 1. INDICELE DE TOXICITATE PE CALE P. O. ȘI I. V. LA ȘOARECE
ȘI NIVELELE SANGUINE LA ȘOBOLAN

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6122

Rezumat. - S-a determinat indicele de toxicitate (DL₅₀) a doi derivați de tiamină cu ciclu tiazole deschis, ditiopropil-tiamina (D.T.P.) și 5-etoxi-carbonil-tiamina (S.E.C.T.), comparativ cu clorhidratul de tiamină. Raportul între DL₅₀ pe cale i.v. și p.o. a fost găsit 1/8 și respectiv, 1/12, în cazul derivaților, față de 1/50 în cazul vitaminei B₁, ceea ce demonstrează resorbția lor superioară pe cale p.o., comparativ cu vitamina B₁.

Urmărind nivelele sanguine după administrarea p.o. a unor doze echimoleculare din toate cele trei substanțe, s-au găsit valori de 20-50 ori mai mari în cazul derivaților decât în cazul vitaminei B₁. Folosind doze chiar de 10 ori mai mici din cei doi derivați, între 15 min și 24 ore, nivelele lor sanguine au fost mai ridicate și de mai lungă durată decât în cazul administrării vitaminei B₁.

Administrarea parenterală a vitaminei B₁ asigură rapid o concentrație utilă în sânge și țesuturi, pe când administrarea p. o. necesită doze foarte mari, datorită resorbției deficitare a vitaminei la nivelul intestinului, și nu realizează niciodată concentrațiile obținute pe cale parenterală.

Această resorbție deficitară este datorită pe de o parte proprietăților fizico-chimice ale vitaminei (liposolubilitate redusă și descompunere sub influența tiaminazei), pe de altă particularităților sale de metabolism, ea resorbindu-se parțial sub formă fosforilată și parțial ca atare.

Organismul are un prag de resorbție pentru forma fosforilată (T.P.F., tiamin-pirofosfat), deoarece nu realizează niciodată depozite de vitamină B₁, excesul fiind eliminat [3], [12], [14].

Administrarea p. o. a dozelor mari de vitamină B₁ arată că de la o anumită doză se obține o plafonare în ceea ce privește resorbția, împiedicând uneori atingerea unor concentrații terapeutice utile [10]. De altfel, raportul între resorbția parenterală și cea orală este ilustrat și de

raportul foarte mic între DL.50 i. v. și DL.50 p. o., care la unele animale are o valoare de cca. 1/40 [12].

În 1952, Fujiwara și Watanabe [5] au izolat pentru prima dată o tiamină cu ciclu tiazolic deschis (alil-tiamina), după care au apărut în literatură numeroși derivați de tiamină care s-au dovedit a avea o resorbție p. o. superioară vitaminei B₁. În acest fel a devenit posibilă realizarea unei medicații eficiente cu vitamină B₁ și pe cale p. o.

Proprietățile chimice comune derivaților cu ciclu tiazolic deschis se referă la: capacitatea lor de a da baze stabile și bine cristalizate; liposolubilitate crescută; lipsa reacției directe a tiocromului în reacția specifică vitaminei B₁ (cu ciclu tiazolic închis) [6].

Din punct de vedere biochimic și farmacologic, acești derivați au câteva caracteristici comune, și anume: se transformă în organism în vitamină B₁ (se reciclează în ficat, fie în prezența agenților reducători de tipul cisteinei sau glutatationului, fie printr-o intervenție enzimatică [6], crescând nivelul cocarboxilazei [6-8] hepatice; se resorb mai bine ca vitamina B₁ pe cale p. o. [1], [6], [9], [10], [15], [16]; au eficacitate de tip vitaminic (experimental și clinic) egală cu cea a vitaminei B₁.

În prezenta lucrare se prezintă rezultatele determinărilor referitoare la indicele de toxicitate acută la șoarece și resorbția în cazul administrării p. o. la șobolani, a 2 derivați de vitamină B₁ cu ciclu tiazolic deschis, diftiopropil-tiamina (D.T.P) și S-etoxi-carbonil-tiamina (SECT).

MATERIAL ȘI METODE

1. S-a determinat la șoarece toxicitatea acută (DL.50) în cazul administrării i. v. și p. o. a produselor. Calculul DL.50 și al limitelor de securitate s-a realizat prin metoda probitului grafic [2]. În cazul administrării i. v., ritmul de administrare a fost lent (1 ml/100 s). S-au folosit șoareci musculi de 18-22 g, loturi de 10 animale pentru o doză.

2. S-au urmărit la șobolani masculi de 120-160 g nivelele sanguine după administrarea p. o. a vitaminei și derivaților, la diferite doze și intervale de timp.

La intervale fixe după administrare (15 și 90 min; 3, 6, 12 și 24 ore) s-a recoltat sânge, din artera carotidă, prin sacrificarea animalelor. Probele de sânge au fost recoltate pe oxalat de sodiu. La aceste probe s-a determinat conținutul în vitamină B₁ totală prin metoda Friedman [4], [11], iar în câteva cazuri și conținutul diferențiat în vitamină liberă și legată. În materialul biologic, în general tiamina și T.P.F. se găsesc legate de proteine. Printr-o extracție în mediu acid, la cald, se realizează hidroliza acestei legături, iar ulterior, în mediu alcalin, se realizează oxidarea tiaminei în tiocrom a cărui fluorescență se citește în u. v. Dozarea vitaminei libere și legate (T.P.F.) s-a efectuat prin determinarea tiocromului înainte și după hidroliza extractelor cu fosfatază. (Fosfataza a fost preparată după Westenbrink [17], din drojdie de bere).

La fiecare doză și interval s-au folosit loturi de minim 5 animale. S-au folosit următoarele substanțe: clorhidrat de tiamină, diftiopropil-tiamină (D.T.P.) și S-etoxi-carbonil-tiamina (S.E.C.T.) sintetizate la I.C.C.F. de un colectiv condus de ing. Mariana Ionescu și ing. Eva Drăgoi. Aceste din urmă substanțe au fost administrate la animale sub formă de clorhidrat preparat extemporaneu. Dozele din fiecare substanță sînt indicate în tabele.

R E Z U L T A T E

1. Toxicitatea acută la șoareci

În tabela 1 sînt indicate valorile DL₅₀ pe diferite căi de administrare și raportul între DL₅₀ p. o. și parenteral în cazul celor 2 produși studiați.

Tabela 1

Toxicitatea acută a derivaților de tiamină cu circa tinsorile de referință

Substanța	DL ₅₀ (mg/kg i.v.)	Limita de securitate	DL ₅₀ (mg/kg p.o.)	Limita de securitate	Raportul DL ₅₀ i.v. / DL ₅₀ p.o.
Clorhidrat B ₁	89,2	84,2—118	8224	6310—10000	1/190
D.T.P. (clorhidrat)	302	279—382	2512	1679—3549	1/8
S.E.C.T. (clorhidrat)	339	296—378	4250	2819—6007	1/12

Din aceste date reiese că:

— Derivații D.T.P. și S.E.C.T. au o toxicitate mai redusă (semnificativă statistic) decît B₁ la administrare i. v. și mai crescută (semnificativă statistic) la administrare p. o.

— Raportul între DL₅₀ i. v și DL₅₀ p. o. indică astfel resorbția p. o. crescută a acestor derivați în comparație cu vitamina B₁.

— Între DL₅₀ ale D.T.P. și S.E.C.T. nu apar diferențe statistice semnificative, dar, atît în cazul administrării p. o. cît și i. v., D.T.P. apare ceva mai toxic decît S.E.C.T.

2. Nivele sanguine după administrare p. o. la șobolan

În tabela 2 sînt indicate rezultatele obținute la 3 ore după administrarea unei doze unice de 500 mg/kg vitamină B₁ (și doze echimoleculare din cei 2 derivați). Din acest tabel reiese că, la 3 ore, nivelul obținut

Tabela 2

Nivele sanguine la 3 ore după administrarea unei doze unice p. o. de 500 mg/kg clorhidrat de tiamină și a unor doze echimoleculare moleculare din cei doi derivați

Substanța	Doza (mg/kg)	Conc. μg/ml sînge tiamină totală	E.S.	Raport concentrație derivați/B ₁
B ₁	500	5,7	± 1,2	—
D.T.P.	550	127,0	± 47	22
S.E.C.T.	550	297,0	± 31	52

cu derivații de B₁ este de 20—50 ori mai mare decît cel obținut cu B₁, folosind doze egale. S.E.C.T. determină nivelele cele mai ridicate (diferențele între D.T.P. și S.E.C.T. nu sînt semnificative statistice).

Urmărind comparativ curba tiaminemiei la diferite intervale de timp (45 minute — 24 ore) datorită diferenței mari între concentrațiile obținute pe de o parte cu B₁ iar pe de altă parte cu cei 2 derivați, dife-

rențe care produc perturbări în metoda de analiză, am folosit din derivații respectivi doze de 10 ori mai mici. În tabela 3 sînt indicate valorile obținute în aceste condiții. Folosind chiar doze de 10 ori mai mici din derivații de vitamină B₁, la toate intervalele între 45 min și 24 ore, nive-

Tabela 3

Nivelele sanguine - în intervale de timp diferite - după administrarea unor doze unice p.o. de vitamină B₁ și de derivați (concentrație în $\mu\text{g/ml}$ sînge tîmînat \pm E.D.)

Timp de la administrare	Vitamină B ₁ (500 mg/kg)	D.T.P. (55 mg/kg)	S.E.C.T. (55 mg/kg)
45 min	2,1 \pm 0,1	3,06 \pm 0,6	31,0 \pm 2,6
90 min	1,9 \pm 0,2	—	—
3 ore	4,3 \pm 0,7	5,75 \pm 3,0	39,0 \pm 8,2
11 2 ore	3,8 \pm 0,2	—	—
6 ore	3,6 \pm 0,4	—	—
12 ore	0,9 \pm 0,03	5,4 \pm 2,5	8,0 \pm 4,6
24 ore	0,7 \pm 0,02	1,4 \pm 0,1	2,4 \pm 0,8

lul sanguin obținut este superior celui obținut cu vitamină B₁. Diferențele sînt semnificative statistic între S.E.C.T. și B₁ la toate intervalele, iar în cazul D.T.P. și B₁ la 12 și 24 de ore.

În cazul administrării S.E.C.T. se obțin, la toate intervalele, nivele sanguine mai ridicate decît cu D.T.P., iar la 24 de ore persistă încă în sînge o cantitate apreciabilă de vitamină, în timp ce în cazul vitaminei B₁ sau al D.T.P. concentrațiile prezente sînt la acest interval sub 1 $\mu\text{g/ml}$.

În cazul tuturor substanțelor curba sanguină este asemănătoare, avînd un maxim în jur de 3 ore. Pentru a urmări capacitatea de esterificare a derivaților, comparativ cu vitamina B₁, s-a efectuat la un număr de probe și o dozare paralelă de tiamină liberă și T.P.F., la 3 ore de la administrarea dozei de 500 mg/kg vitamină B₁ și 55 mg/kg S.E.C.T. și D.T.P. Rezultatele sînt trecute în tabela 4.

Tabela 4

Nivelele sanguine - la 3 ore - după administrarea p.o. a unei doze unice de vitamină B₁ și derivați (concentrație în $\mu\text{g/ml}$ sînge)

Substanța	Doza (mg/kg)	Tiamină liberă	Tiamină legată	Tiamină totală	% tiamină legată
B ₁	500	0,1	2,4	2,5	96
	500	0,1	1,4	1,5	93
D.T.P.	55	0,8	7,7	8,5	90
	55	1,9	5,1	7,0	72
S.E.C.T.	55	17	32	49	65
	55	19	30	49	51

Aceste rezultate reduc puțin din diferența mare găsită între concentrațiile sanguine eficiente de B₁ și de derivați, în special în cazul S.E.C.T., știut fiind că T.P.F. — forma legată — reprezintă partea activă a vitaminei. Dar și în aceste condiții se păstrează superioritatea derivaților cercetați față de B₁.

DISCUȚIA REZULTATELOR

În ceea ce privește D.T.P., există numeroase cercetări în literatură care arată superioritatea acestui derivat față de vitamina B₁ în cazul administrării lui per orale [6-9]; în ceea ce privește însă S.E.C.T., substanță sintetizată inițial de autorii japonezi, cercetările farmacologice sînt foarte reduse [15]. Nu se găsesc în literatură studii comparative asupra toxicității sau resorbției acestor doi derivați, în așa fel încît să se poată întrevădea superioritatea unuia sau altuia în vederea întrebuirii lor în terapiesc.

Cercetările farmacologice prezentate ne-au permis în primul rînd să confirmăm intensă resorbție per orală a acestor derivați, comparativ cu vitamina B₁, manifestată atît prin nivelul sanguin crescut cît și prin raportul avantajos între indicele de toxicitate pe cale p. o. față de calea i. v.

În cazul determinărilor de toxicitate, valorile găsite pentru DL₅₀ ale vitaminei B₁ și D.T.P. sînt corespunzătoare datelor din literatură [13], [17]; de asemenea, concentrațiile de tiamină totală în sînge, găsite de noi, se apropie de cele găsite de Hiroco și colab. [7], [8]. În ceea ce privește S.E.C.T., datele din literatură se referă numai la nivele sanguine la iepuri și om, care și în cazul autorilor japonezi sînt mult mai ridicate decît cele obținute după administrarea unor doze egale de vitamină B₁ [15].

Urmărind raportul între concentrațiile sanguine găsite în cazul administrării S. E. C. T. și D. T. P. la șobolan, reiese superioritatea S.E.C.T. și posibilitatea obținerii unor nivele sanguine de cea 6 ori mai mari cu această substanță, comparativ cu D. T. P. Trebuie însă să ținem seama de posibilitatea existenței unor diferențe între speciile de animale în ceea ce privește resorbția per orală, metabolizarea, posibilitatea de esterificare etc. a acestor derivați și, din acest punct de vedere, rezultatele obținute pe șoareci (unde toxicitatea pe cale p. o. a S. E. C. T. este mai redusă ca a D. T. P.) pledează pentru o mai redusă resorbție a S. E. C. T. la șoarece decît la șobolan.

De asemenea, nu putem să transpunem direct, de la animale la om, superioritatea S. E. C. T. asupra D. T. P., întrucît există și o diferență în ceea ce privește gradul de esterificare a celor doi derivați chiar la șobolan, D. T. P. fiind esterificat (legat) într-o proporție mai însemnată decît S. E. C. T. Cercetări ulterioare vor lămurii aceste aspecte, în vederea stabilirii valorii terapeutice a celor doi derivați pentru clinică.

CONCLUZII

1. Derivații de vitamină B₁ cu ciclu tiazolic deschis, D. T. P. și S. E. C. T., au un indice de toxicitate mai crescut decît vitamina B₁ în cazul administrării i. v., dar mai scăzut în cazul administrării p. o. la șoarece.

2. D. T. P. și S. E. C. T. determină nivele sanguine mult mai ridicate și de durată, comparativ cu vitamina B₁, în cazul administrării lor p. o. la șobolan.

3. Din cercetările efectuate la animal apar următoarele diferențe cantitative între D. T. P. și S. E. C. T.: toxicitatea S. E. C. T. p. o. la șoarece este mai redusă decât a D. T. P.; nivelele sanguine obținute după administrarea p. o. a S. E. C. T. la șobolan sînt mai ridicate la toate intervalele între 45 de minute și 24 de ore, decât după administrarea D. T. P.; la 3 ore de la administrare, S. E. C. T. sub formă legată se găsește într-o proporție mai mică decât după administrarea D. T. P.

4. Derivații de tiamină cu ciclu tiazolic deschis, D. T. P. și S. E. C. T., derivați de vitamină B₁ cu eficacitate egală, dar cu resorbție superioară în cazul administrării lor per orale, apar ca substanțe de largă perspectivă pentru viitor, alături ca medicamente de uz uman, care permit o administrare eficientă, dar mai lesnicioasă (p. o.) de vitamină B₁ cît și ca biostimulatori pentru uz zooveterinar, realizîndu-se din acest punct de vedere concentrații mari de vitamină în hrană (furaje).

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București

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THERAPEUTIC USE OF VITAMIN B₁ IN DISEASES OTHER THAN BERIBERI

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It must have been a fascinating experience for physicians in 1936 and the following years to observe the therapeutic action of vitamin B₁ in severe beriberi. The incapacitating signs of polyneuritis and the life-threatening cardiovascular decompensation disappeared in a few days after the first thiamine injection. Grossly enlarged hearts regained normal size, massive edemas were eliminated, and intractable pains vanished. It must have been equally fascinating for the biochemists who subsequently discovered a large number of biochemical reactions in the intermediary carbohydrate metabolism for which thiamine was an essential part of the catalyzing enzymes. The enthusiasm of the vitamin pioneers in underdeveloped countries and the biochemists at home was not without influence on us physicians who are privileged to practice in well-nourished countries where a true case of beriberi would be a sensational rarity. It is thus understandable that we were and still are giving this vitamin to patients whose symptomatology is similar to beriberi, whose dietary history is suggestive of a certain degree of vitamin B deficiency or who have disturbances of the carbohydrate metabolism.

Indications for Vitamin B₁ Therapy

In order to determine the diseases, syndromes and symptoms for which vitamin B₁ has been used in treatment, we screened the *Current List of Medical Literature*, the *Quarterly Cumulative Index Medicus* and our own literature files, and have listed all medical papers whose titles specifically mentioned a therapeutic use of vitamin B₁ for a particular disease in human subjects. In this way, 696 published papers reporting vitamin B₁ therapy in more than 230 different diseases and syndromes have been found. It is interesting to note that two thirds of these papers were by European investigators, the rest being equally divided between investigators of the United States and the rest of the world. FIGURE 1, which shows not only the distribution of the papers as to origin but also the years of publication, clearly indicates two periods of major interest in vitamin B₁, namely the years from 1938 to 1940 and, to a somewhat lesser degree, from 1952 to 1955.

It would not serve any useful purpose to list all the conditions for which vitamin B₁ has been tried as the sole therapeutic agent or in combination with drugs. It may however be interesting to compare the main indications in the first decade of clinical experience with those between 1951 and 1960. From the number of published papers summarized in TABLE 1 it can be seen that the symptoms resembling beriberi, e.g. neuritis, neuralgias, paralyses, pains of various origin, and diseases of the central nervous and cardiovascular systems were the most important targets for vitamin B₁ therapy between 1936 and 1945. In recent years, metabolic aberrations, e.g. acidosis, diabetic coma, pyruvemia, and toxemia of pregnancy became the more prevalent indications.

Present Day Use of Vitamin B₁ in Medical Practice

We were interested to learn which of the many therapeutic indications explored or proposed for vitamin B₁ had in the many years of practical medical experience proven to be of real use to the physician. A questionnaire was therefore sent to a number of neurologists, internists, pediatricians, and representatives of other specialties selected at random throughout continental United States. The survey included questions about actual use and indications for vitamin B₁ in the physician's practice, route of administration, drugs and vita-

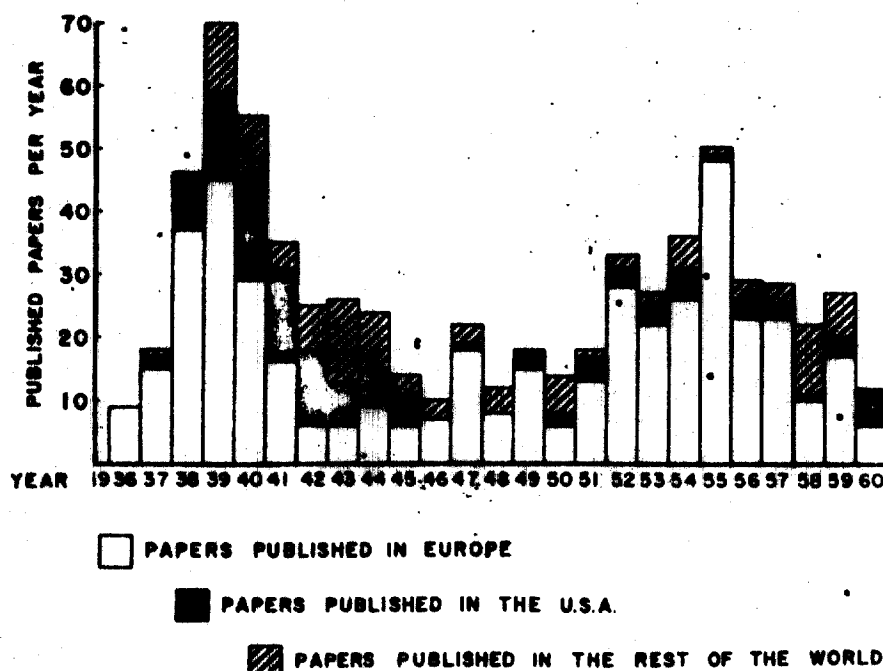


FIGURE 1. Papers published on the therapeutic use of vitamin B₁ in diseases other than beriberi.

mins used in combination with vitamin B₁, desired action, and thoughts about overuse or misuse of vitamin B₁.

Results

One hundred and fourteen, or 23 per cent, of the physicians contacted answered the questionnaire. Eighty-five per cent of the neurologists, 72 per cent of the internists, and 81 per cent of the group including various specialties are regularly prescribing vitamin B₁, whereas only 36 per cent of the pediatricians make use of this compound. Over 70 diseases and symptoms were listed as indications for vitamin B₁ therapy; the ones most frequently mentioned are summarized in TABLE 2. As expected, peripheral neuropathy heads the list. Of 27 neurologists, for example, only four are not prescribing vitamin B₁ for this indication, one of them because of lack of suitable patients. It is further

TABLE 1
INDICATIONS FOR VITAMIN B₁ THERAPY IN HUMANS AS
REFLECTED IN THE SCIENTIFIC LITERATURE*

Indication	Number of published papers		
	1936-1960	First decade 1936-1945	Last decade 1951-1960
Peripheral neuritis (unspecified)	38	27	9
Neuralgias (trigeminal, sciatica, lumbago, ampu- tation stump)	28	16	4
Pain of various origin (labor, postoperative, head- ache, etc.)	28	7	13
Diabetes mellitus	21	11	6
Cardiac decompensations, cardiopathies	20	6	14
Rheumatic pains	15	4	9
Postdiphtheric paralysis	13	11	0
Cardiovascular diseases (unspecified)	13	5	6
Herpes zoster	13	8	2
Diabetic coma	13	0	9
Toxemia of pregnancy	12	1	11
Mental disorders, depression, psychosis	11	5	5
Funicular myelosis in pernicious anemia	10	9	0
Hypervitaminosis (of pregnancy)	10	6	3
Acidosis (in diabetes and other)	9	1	7
Peripheral vascular diseases	9	5	4
Multiple sclerosis	9	1	8
Purpura (postsurgical, in pregnancy, in pre- matures)	9	0	9
Labor acceleration	9	0	8
Delirium tremens	8	7	1
Tabetic pains	8	7	0
Neural leprosy	8	7	1
Radiation sickness	7	5	2
Mental deficiency	7	1	4
Organic CNS disease (unspecified)	7	4	2
Neuritis in pregnancy	6	5	0
Acrodynia	6	5	0
Poliomyelitis	6	4	1
Prophylaxis of thromboembolism	6	0	6
Diabetic polyneuritis	5	3	1
Alcoholic polyneuritis	5	4	0
Varicella	5	1	4
Shock (unspecified)	5	4	1
Anemia	5	1	4
Wernicke's encephalopathy	5	0	5
Thyrotoxicosis	4	3	1
Retrobulbar optic neuritis	4	2	0
Paralytic ileus	4	4	0
Disorders of the autonomic nervous system	4	1	3
Leg ulcers	4	2	2
Deafness, impairment of hearing	4	4	0
Infant toxicosis	4	0	4
Tuberculosis	4	0	4
Posttraumatic edema	4	0	4
Cranial trauma	4	0	4
Migraine	4	2	1

* 143 additional indications with less than 4 publications are not listed.

evident that in peripheral neuropathies of various origins parenteral administration is preferred over oral, and combinations with vitamin B complex or vitamin B₁₂ are more frequently used than the vitamin B₁ alone.

TABLE 2
THE MOST IMPORTANT INDICATIONS FOR VITAMIN B₁ THERAPY ACCORDING TO A
PHYSICIAN'S SURVEY MADE IN 1961*

Indication	Number of physicians prescribing vitamin B ₁	Preferred route of administration		Vitamin B ₁ alone	In combination with							
		Oral	Parenteral		Vit. B complex	Multi-vitamin formulations	Vitamin B ₁₂	Prednisolone	Salicylates	Iron	Vitamin C	Miscellaneous drugs
Peripheral mono- or polyneuritis	42	20	24	7	14	4	13	1	1	—	—	—
Diet supplement and general prophylaxis	15	9	2	0	1	10	—	—	—	—	—	—
Anemia	14	9	3	2	3	4	2	—	—	3	2	—
Wernicke's encephalopathy	12	0	8	3	3	1	—	—	—	—	—	1
Vitamin deficiencies	11	7	4	3	1	4	—	—	—	—	—	—
Malnutrition	10	7	5	1	—	5	—	—	—	—	—	—
Chronic alcoholism	8	6	5	2	3	—	—	—	—	—	1	—
Alcoholic neuritis	8	3	4	1	1	2	—	—	—	—	—	—
Beriberi	5	2	4	2	1	1	—	—	—	—	—	—
Chronic debility and illness	5	3	2	1	4	2	1	—	—	—	2	—
Cardiac decompensation	5	4	4	1	—	—	—	—	—	—	—	—
Gastrointestinal disorders	5	2	2	—	2	1	1	—	—	—	—	—
Pre- and postoperative use	4	2	3	—	1	1	—	—	—	—	—	—
Diabetic neuropathy	4	2	2	1	3	—	2	—	—	—	1	—
Neuralgia	4	1	2	2	2	—	1	—	—	—	1	—
Herpes zoster	4	1	3	1	—	—	1	1	1	—	—	—
Multiple sclerosis	4	1	2	—	1	—	1	—	—	—	—	2
Nutritional anemia	3	1	2	—	—	—	1	—	—	2	—	1
Delirium tremens	3	—	3	2	—	—	—	—	—	—	—	—
Vertigo and tinnitus	3	—	—	—	—	—	—	—	—	—	—	2
Diabetes mellitus	3	3	1	1	1	—	—	—	—	—	—	—
Insect repellent	3	2	—	3	—	—	—	—	—	—	—	—

* Thirty-six additional indications with less than 3 physicians prescribing are not listed.

Nutritional indications—general prophylaxis, anorexia, malnutrition and vitamin deficiencies with vague symptoms and complaints—are also frequently mentioned; in these the oral administration is definitely preferred and vitamin combinations are logically given more frequently than vitamin B₁ alone. Next on the list are chronic alcoholism and its complications, such as Wernicke's en-

cephalopathy and peripheral neuritis. Farther down the line we find various indications which may have received a more prominent position 20 years ago, but have since become less important in this country due to the improvement of general nutrition, vitamin enrichment of food, vaccination, and other preventive measures. This is particularly true for cardiac and gastrointestinal disturbances occurring as a consequence of malnutrition or imbalanced diets and postdiphtheric paralysis. It is quite interesting that vitamin B₁ is frequently prescribed in combination with other vitamins but very rarely with drugs. This seems to prove that most physicians reserve this vitamin for those cases in which the dietary history is highly suggestive of a nutritional deficiency, and that indiscriminate use of vitamin B₁ is the exception. This can also be seen from the reply to our question as to whether vitamin B₁ was thought to be overused or misused in certain indications. Only a few physicians had any comments, anorexia (10 times) and emotional disturbances (9 times) being mentioned most frequently.

Pharmacology of Vitamin B₁

Could some of the therapeutic actions which have been suggested or claimed for vitamin B₁ be explained by the pharmacologic properties of this agent? In a review of this subject in 1954 Unna²⁸ points out that only very high doses of thiamine cause pharmacologic effects, such as depression of the respiratory center, transient fall in blood pressure, depression of ganglionic transmission,⁴ a curarelike action at the neuromuscular junctions,⁴ and bronchoconstriction and inhibition of cholinesterase. Bradycardia and peripheral vasodilatation have also been reported.^{29,30} In order to reinvestigate this problem, vitamin B₁ was submitted under a code number for basic pharmacologic screening in our Pharmacology Department. The pharmacologists were informed only about the LD₅₀ of the substance and were asked to conduct a preliminary screening for interesting pharmacologic actions as is routinely done with many other compounds. The following screening tests were performed after administration of various doses of vitamin B₁ as shown in TABLE 3.

- (1) Acute toxicity, determination of LD₅₀ and lethal effects in mice.
- (2) Muscle relaxant effect. Determination of the dose causing paralysis in 50 per cent of the animals (PD₅₀) in the inclined screen test in mice.³¹
- (3) Fighting mouse test. Determination of dose which suppresses fighting episodes in electrically stimulated mice.³²
- (4) Anti-inflammatory effects. Inflamed foot test of Randall and Selitto:³³
 - (a) reduction of edema in rat's foot induced by subcutaneous infection of yeast,
 - (b) temperature change in inflamed foot and noninflamed foot, and (c) increase of pain threshold in inflamed and noninflamed foot.
- (5) Analgesic effect. Rat tail method of D'Amour-Smith.⁴
- (6) Diuretic effect determined in dogs.
- (7) Anticonvulsant effects: (a) minimal electroshock convulsant threshold,³⁴ (b) maximal electroshock seizures,³⁵ and (c) pentamethylenetetrazole test.⁴
- (8) Effect on appetite: (a) determination of weight gain in rats in comparison with untreated animals during a 4-hour feeding period, and (b) determination of food consumption in comparison with untreated animals during a 4-hour feeding period.

TABLE 3
PHARMACOLOGIC SCREENING RESULTS WITH THIAMINE HYDROCHLORIDE

Screening test	Animal	Result
1. Acute toxicity	Mouse	LD ₅₀ P.O. about 3000 mg./kg. I.V., 100-125 mg./kg. excitation; Straub tail phenomenon at 200 mg./kg. I.P.; death by respiratory failure at 500 mg./kg. I.P.
2. Muscle relaxant effect	Mouse	PD ₅₀ > 500 mg./kg. P.O.
3. Fighting mouse test	Mouse	ED ₅₀ > 100 mg./kg. P.O.
4. Anti-inflammatory action at 50 mg./kg. s.c. (a) antiedema effect (b) temperature change (c) increase of pain threshold	Rat	4% Inflamed foot: +0.5° C., noninflamed foot: +0.9° C., rectal: -0.2° C. Inflamed foot: 16% noninflamed foot: 40%
5. Analgesic effect	Rat	No analgesia at doses up to 1500 mg./kg. P.O.
6. Diuretic effect	Dog	No significant increase of water and Na ⁺ excretion at 8 and 25 mg./kg. I.V.
7. Anticonvulsant effect (a) minimal electroshock convulsant threshold (b) maximal electroshock seizures (c) antipentylenetetrazole	Mouse	ED ₅₀ > 800 mg./kg. P.O. ED ₅₀ > 800 mg./kg. P.O. ED ₅₀ > 800 mg./kg. P.O.
8. Effect on appetite	Rat	No effect on weight gain and food consumption at 50 mg./kg. s.c.
9. Gastric secretion	Dog	No effect on gastric secretion and acidity at 4, 16 and 64 mg./kg. I.V.
10. Antiulcer effect (a) Shay rat method (b) histamine ulcers	Rat. Dog	No effect at 4, 16, 64 and 128 mg./kg. s.c. No effect at 4, 16, 64 and 128 mg./kg. s.c.
11. Intestinal tone and motility (a) unanesthetized dog (b) anesthetized cat	Dog Cat	No effect on jejunal tone or motility at 4, 8 and 16 mg./kg. No effect on jejunal tone and motility at 0.5, 1, 2 and 4 mg./kg.; partial inhibition of serotonin-induced spasm
12. Bile flow	Dog	No effect at 16 and 64 mg./kg. I.V.
13. Antitussive effect (a) electrically induced cough (b) cough induced with ammonia	Dog Cat	No effect at 4, 16, 36 and 64 mg./kg. P.O. and I.V. No effect at 64 and 128 mg./kg. P.O. and I.V.
14. Sialoschesis	Rabbit	No effect on pilocarpine-stimulated saliva flow at 4 and 32 mg./kg. s.c.
15. Mydriasis	Rabbit	1, 5, and 10% solution applied to conjunctival sac without effect
16. Antihypertensive action	Dog, hypertensive	No significant effect on blood pressure at 16 and 64 mg./kg. I.V.
17. Cardiovascular screening at 4 mg./kg. (a) mean blood pressure (b) response to serotonin (c) response to carotid artery occlusion	Dog	Blood pressure fall 14 mm. Hg 3 min. duration No change No change

TABLE 3—Continued

Screening test	Animal	Result
(d) response to hypertensin (e) response to norepinephrine (f) response to cranial vagus nerve stimulation (g) response to histamine		No change No change No change No change
18. Cardiovascular screening at 1, 2 and 4 mg./kg. (a) blood pressure and respiration (b) peripheral vagal stimulation (c) bilateral carotid artery occlusion (d) response to norepinephrine	Dog, biva- gotomized	Prolongation of recovery period of blood pressure following histamine No significant change No effect on cardiac inhibition, ventricular escape and cardiac acceleration No effect on cardiac acceleration and blood pressure rise Slight potentiation of cardiac response and partial inhibition of vasoconstrictor effects
19. Cardiovascular screening in chloralose cats at 0.5, 1, 2 and 4 mg./kg. I.V.. (a) blood pressure (b) postural effect (c) response to acetylcholine (d) response to serotonin (e) response to norepinephrine	Cat	No effect Following 2 and 4 mg./kg. temporary improvement of sympathetic compensatory pressor effect and increase of cardiac acceleration No effect Inhibition of bradycardia for short periods following the larger doses Some inhibition of cardiac response and the peripheral vascular vasoconstrictor effect
20. Effect on nictitating membrane (a) preganglionic stimulation (b) membrane response to I.V. serotonin	Cat	Following 4 mg./kg. I.V. the contractile amplitude appeared reduced to 66% of the original effect Unchanged
21. Isolated auricle, effect on postextrasystolic potentiation of the basic contraction	Cat	1, 4, and 16 γ /ml. depressed contractile force (negative inotropic action); 64 γ /ml.: postextrasystolic potentiation equivalent to that of normal tissue; 256 γ /ml.: negative inotropic effect
22. Animal behavior (Sidman avoidance procedure)	Rat	25 mg./kg. and 50 mg./kg. I.P. without effect
23. Antiparkinsonism test	Monkey	2 mg./kg. s.c. without effect on parkinsonism-like syndrome
24. Aminoxidase inhibition	In vitro	No inhibition at 10^{-3} M
25. DOPA decarboxylase	Rat	100 mg./kg. I.P. caused 25.2% inhibition
26. Serum cholesterol	Rat	No effect at 50 mg./kg./day s.c.
27. Blood sugar at 100 mg./kg. P.O.	Rat	Blood sugar after 2 hr. +1% after 4 hr. +4%
28. Potentiation of 5-HTP	Mouse	No effect at 100 mg./kg. I.P.
29. Potentiation of DOPA	Mouse	No effect at 100 mg./kg. I.P.

- (9) Gastric secretion in Heidenhain pouch¹⁰ dog with and without histamine stimulation.
- (10) Antiulcer effect: (a) Shay rat method,²⁴ and (b) histamine-induced ulcers in dogs.²⁵
- (11) Intestinal tone and motility: (a) tested in unanesthetized Thiry-Vella loop dogs, and (b) tested in chloralose cats with and without serotonin-induced spasm.
- (12) Effect on bile flow tested in cholecystectomized dogs.
- (13) Antitussive effect: (a) tested in unanesthetized dogs, cough reflex induced by electrical stimulation of the trachea,²⁶ and (b) tested in anesthetized cats, cough reflex induced by insufflation of ammonia in the airways.²¹
- (14) Sialoschesis. Determination of saliva flow in unanesthetized rabbits after pilocarpine stimulation.
- (15) Mydriasis. Vitamin B₁ was applied to the conjunctival sac of rabbits.
- (16) Antihypertensive action tested in unanesthetized carotid loop dogs with Goldblatt hypertension.
- (17) Cardiovascular screening in nembutalized dogs: (a) mean blood pressure, (b) response to serotonin (25 γ /kg.), (c) response to carotid artery occlusion (15 sec.), (d) response to hypertension (0.5 γ /kg.), (e) response to norepinephrine (1 γ /kg.), (f) response to cranial vagus nerve stimulation, and (g) response to histamine (5 γ /kg.).
- (18) Cardiovascular screening, nembutalized, bilaterally vagotomized dog: (a) blood pressure response and respiration, (b) response to peripheral vagal stimulation, (c) response to bilateral carotid artery occlusion, and (d) response to norepinephrine.
- (19) Cardiovascular screening in chloralose-cats: (a) blood pressure response, (b) postural effects (response to tilting 45° for 30 sec.), (c) response to acetylcholine (1 γ /cc.), (d) response to serotonin (25 γ /kg.), and (e) response to norepinephrine (1 γ /kg.).
- (20) Effect on nictitating membrane in chloralose-cats: (a) preganglionic stimulation, and (b) membrane response to serotonin.
- (21) Effect on isolated auricle (cat). Electrical stimulation of cardiac tissues at 30 cycles/min. Introduction of extrasystoles at 100–1000 msec. following basic contraction. Observation of drug effect on postextrasystolic potentiation.
- (22) Behavior effect in the nondiscriminated (*Sidman*) avoidance procedure of rats.²⁷
- (23) Antiparkinsonism test: observation of drug effect on reserpine-induced parkinsonismlike syndrome in monkeys.²¹
- (24) Monoamine oxidase inhibition *in vitro*.²⁸
- (25) DOPA decarboxylase inhibition *in vivo*.
- (26) Effect on serum cholesterol in rats. Vitamin B₁ administered for two weeks.
- (27) Effect on blood sugar in rats.
- (28) Potentiation of 5-hydroxytryptophan in mice.²⁹
- (29) Potentiation of dihydroxyphenylalanine (DOPA) in mice.²⁹

Results

As indicated in TABLE 3 pharmacologic screening of vitamin B₁ yielded essentially negative results. There were some weak cardiovascular effects, such as transient blood pressure fall, improved sympathetic compensation following postural tilting, negative inotropic effects on isolated auricular tissue, temporary block of bradycardial response to serotonin and possibly a prolongation of the vascular recovery following histamine. Furthermore, partial reduction in nicotinic membrane response to preganglionic stimulation and a partial inhibition of the serotonin-induced intestinal spasm were observed. None of these effects was sufficiently pronounced to account for a therapeutic action.

Mode of Action of Vitamin B₁ as a Therapeutic Agent

In most of the papers reporting therapeutic results with vitamin B₁, the double blind technique, crossover design, or other measures of control were not employed. In addition, the literature contains conflicting opinions as to the therapeutic efficacy of this vitamin for almost all diseases and symptoms for which it has been tried. Nevertheless, it would be inappropriate to disregard the hundreds of clinical reports, observations and testimonials describing the therapeutic effectiveness of vitamin B₁, and an attempt shall therefore be made to summarize the factors which could account for the vitamin B₁ action in diseases other than severe beriberi.

From all we know about the pharmacology of vitamin B₁, it is unlikely that at the doses used in human subjects it could exert a therapeutic action, by virtue of its pharmacodynamic properties. There may be two exceptions where thiamine, as such, is directly responsible for a physiological action, namely in the rare instances of thiamine shock which is thought to be an anaphylactic reaction,^{11,24,25} and possibly when used as an insect repellent; the latter has been claimed by various observers but could not be proved under controlled laboratory conditions.^{17,18}

In other instances, the therapeutic effect of vitamin B₁ may be explained by the fact that some symptoms for which treatment was given are due to a degree of vitamin B₁ deficiency. In most cases this is very difficult to prove because vitamin B₁ excretion and thiamine, pyruvate, and lactic acid blood levels have only limited diagnostic value. However, there are clinical reports available of patients whose dietary history was suggestive of a vitamin deficiency and in whom thiamine administration was followed by a prompt therapeutic response.^{1,26} In most instances, malnutrition means deficiency of all or most essential vitamins, proteins, fat, and even carbohydrates, and it is often impossible to determine which of the multiple symptoms can be attributed to vitamin B₁ deficiency. Thus the vitamin must often be given without specific diagnosis.

In our day of vitamin enrichment of food, the cases of marked hypovitaminosis are infrequent. There is evidence, however, that vitamin B₁ deficiency plays an important role in the pathogenesis of various complications of chronic alcoholism. There is no reason to believe that high alcohol intake, as such, causes vitamin B₁ deficiency, and carboxylase does not seem to be involved in the metabolism of ethanol. Careful studies by Joliffe and collaborators²⁷ have shown that malnutrition is the basic cause for the neurological complica-

tions of chronic alcoholism. Although it is certainly true that the diet of most alcoholics is deficient in many respects and that liver disease and gastrointestinal disturbances may become contributing factors, the prompt action of vitamin B₁ injections, particularly in alcoholic neuritis, certain symptoms of Wernicke's encephalopathy and cardiac decompensation, suggests that vitamin B₁ deficiency is the most important cause of these complications.

Particularly in Europe, high doses of vitamin B₁ are almost routinely used in various forms of neuralgias and myalgias, and very promising results are claimed by many careful investigators. It has been speculated that because of its importance for the conduction of nerve impulses,¹⁸ vitamin B₁ may influence various forms of pain, even in patients without nutritional deficiency. This opinion is not shared by the majority of the American neurologists who believe that only those cases of neuritis which are due to vitamin B₁ deficiency respond to vitamin therapy.

In recent years, the direct metabolic action of vitamin B₁ and especially its pyrophosphoric acid ester (cocarboxylase) has been discussed extensively, a problem recently reviewed by Segre.²² Increased serum pyruvate and lactic acid levels have been found in many pathologic conditions, such as severe kidney and liver diseases, diabetic acidosis, various forms of toxicosis, cardiac decompensation, digitalis intoxication, acetonemic vomiting, myocardial infarct, radiation therapy, pneumonia, epilepsy, after narcosis and surgery, and so on. Administration of cocarboxylase, sometimes also of thiamine hydrochloride, has, according to many reports, brought about a prompt normalization of the lactic and pyruvic acid levels in blood and urine.^{12,18,40} In other studies, no effects of cocarboxylase on diabetic acidosis and pyruvic acid levels in animals and man were found.^{7,20,27} Thus it seems that the therapeutic effect of the vitamin and the coenzyme largely depends on the cause of the acidosis. It has been speculated that the disturbances of the pyruvate metabolism may be due to vitamin B₁ deficiency or impaired phosphorylation of thiamine.^{9,14} In the heart in particular, hypoxia may lead to a loss of energy-rich phosphates and thus an impaired synthesis of cocarboxylase.¹ In such cases, therapy with cocarboxylase would be justified. On the other hand, the protein part of the carboxylase may also be inhibited or inactivated, leading to an acidosis not influenced by vitamin B₁ or cocarboxylase.⁷

Is the normalization of the pyruvate and lactic acid levels of any significance for the symptomatology of the underlying disease? Many clinical papers report prompt cessation of various symptoms after pyruvate and lactate blood levels have been normalized, particularly in cardiac decompensation, arrhythmias, digitalis intoxication, myocardial infarction,^{1,2,9,24} and radiation therapy.²⁵ Furthermore, improved response of the heart muscle to digitalis and strephantine^{2,16,26} has been reported. Adequate control of such largely subjective symptoms is very difficult and in many cases impossible.²² However, extensive clinical impressions published in the past few years would suggest that adjuvant therapy with cocarboxylase or thiamine hydrochloride in acidosis of various origins may be considered, particularly in cases in which malnutrition, impaired intestinal absorption and vomiting, or a diet rich in carbohydrates, could have caused a relative carboxylase deficiency.

Summary

In the past 25 years, vitamin B₁ has been tried as a therapeutic agent in several hundred diseases and syndromes. During this time, extensive clinical research and broad experience in daily practice with this vitamin have led to a satisfactory determination of its usefulness and indications. It appears that peripheral neuritis is the most important target for vitamin B₁ therapy, the chance for therapeutic success being best in cases in which malnutrition, nutritional imbalance, vomiting, and impaired intestinal absorption are present. Thus neuropathies due to avitaminosis, chronic alcoholism, and pregnancy definitely represent absolute indications for vitamin B₁. The pharmacologic evaluation of thiamine has not uncovered any pharmacodynamic activities likely to serve as a basis for therapeutic use. Thus the clinical application of vitamin B₁ is restricted to such conditions as are at least partially due to hypovitaminosis. Fortunately, improved nutrition and vitamin enrichment of food have greatly diminished the incidence of hypovitaminosis in many countries. If B₁ hypovitaminosis occurs, however, the symptomatology may be multifiform, due to the position of vitamin B₁ as coenzyme in carbohydrate metabolism; in such cases, results of the vitamin therapy are often truly spectacular. The use of vitamin B₁, particularly in the form of the pyrophosphoric acid ester (cocarboxylase) in acidosis of various origins is a new indication which is still controversial. However, the biochemical background and the clinical facts already on hand seem to justify further investigation.

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THE PROBLEM OF THE EFFECT OF VITAMIN B₁ ON CONDITIONED REFLEX ACTIVITY IN DOGS

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In a previous paper (1) we showed that the administration (subcutaneous) to healthy dogs of vitamin B₁ in doses of 2 and 5 mg during the course of 11-13 days caused no sort of discernible changes on the part of conditioned reflex activity, but that administration in 10- and 20-mg doses over 20 days led to changes in the functional state of the cortex of the large hemispheres, which are reflected in a weakening of conditioned reflex reactions, their instability, the disruption of force behavior, etc. The administration of vitamin B₁ per os, in doses of 50 and 100 mg over 10 days did not lead to any sort of discernible disruption in cortex functions of the large hemispheres.

The aim of the present work was to explain the effect of large doses of vitamin B₁ on the conditioned reflex activity of healthy dogs, and in particular on limited inhibition caused by the use of a high-power conditioning stimulus. The experiments were conducted on two dogs (Usnik and Tsezor) using the method of food-conditioned reflexes.

The conditioned reflexes in the dogs were developed with the following stimuli: a bell, a light, a tone (66 and 119.5 dB loudness), gurgle + and gurgle - (differentiation). It is possible to attribute to the animals, both Usnik and Tsezor, a strong, steady type of nervous system. In both dogs, at the beginning, conditioned reflexes were formed to the 66-dB tone, and then immediately but with gradual transition, to the 119.5-dB tone. On this basis, the experiments were conducted with the vitamin. Vitamin B₁ was administered to Usnik by means of subcutaneous injection in doses of 100 and 150 mg. Vitamin B₁ was given to Tsezor in milk, in 500-mg doses. The results of the experiment are presented in Tables 1 and 2 following.

Table 1

Mean Values of Conditioned Reflexes in the Dog Usnik with Subcutaneous Administration of Vitamin B₁ in 100- and 150-mg Doses (at 6-Day Intervals)

Cond.g Stim.	With 66-dB Tone (Exp. # 539-42)	With 119.5-dB Tone (Exp. # 546-551)	Admin. of 100-mg Vit. B ₁ (Exp. # 557-562)	With Admin. of 150-mg Vit. B ₁ (Exp. # 565-570)	After end of Vit. B ₁ Admin. (Exp. # 576-581)
Bell	62	61	56	56	62
Light	62	54	47	46	55
Tone	44	36	37	34	35
Gurgle +	59	44	46	39	47
Gurgle -	4	8	5	9	6
Bell	45	49	42	45	50
Total	272	244	228	220	249

As is evident from Table 1, reflex to a tone of 119.5 dB, in comparison to the reflexes to the remaining stimuli in the system, is insignificant. Thus, the increase in tone loudness from 66 to 119.5 dB caused the development of a not-sharply expressed limited inhibition to this tone. This limited inhibition

exerted its influence on the size of the reflex to light and to gurgle + as well. Correspondingly, the total of conditioned reflexes also decreased. With this background, 100 mg of vitamin B₁ were administered daily to the dog for 13 days. The administration of the vitamin, starting from the 5th

Table 2

Mean Size of Conditioned Reflexes in the Dog Trezor with Administration of Vitamin B₁ per os in 500-mg Doses (at 6-Day Intervals)

Cond.g Stim.	With 66-dB Tone (Exp. #481-486)	With 119.5-dB Tone (Exp. # 488-9,492-5)	With Admin. of 500-mg Vit. B ₁ (Exp. 10th day of admin., exp. # 507-512)	With Admin. of 500-mg Vit. B ₁ (at end of exp: #513-518)	After End of Vit. B ₁ (Exp. # 519-524)
Tone	29	19	14	10	17
Touch	51	34	31	22	30
Gurgle +	26	24	23	20	22
Gurgle -	4	4	6	7	4
Tone	20	13	9	5	12
Touch	35	26	23	19	29
Total	161	116	100	76	100

day of its use, caused a lowering of the size of conditioned reflexes to the bell and light, in comparison with the size of these reflexes before the beginning of vitamin administration (see Table 1). The size of the reflexes with daily administration of 150-mg of vitamin B₁ in the course of 11 days was at approximately the same level. But there was sometimes observed a lengthening of the delay period, a certain unsteadiness in conditioned reflex reactions, drowsiness of the animal during the experiment, and hypnotic phases - levelling and paradoxical (experiments 566 and 570, Table 3).

Table 3

Usnik; 150 mg Vitamin B₁ Administered

Time	Rank #	Cond.g Stim.	Disch. Time, sec	Delay Per., sec	Cond.d Refl. Value in scalar units	Digest. Move.t Rcn.	Cond.d Refl. Value aft. every 5 sec in scalar units	Uncond.d Refl. Value aft. 1 min
Experiment # 566, Jan. 21, 1955								
3h.10m.	143	Bell	20	2	42	I, II	5-7-12-18	462
23	292	Light		4	49		7-11-12-19	443
28	309	119.5- dB tone		4	49		2-4-4-9	416
33	306	Gurgle +		3	44		9-11-12-12	-
38	281	Gurgle -			6	I, -	3-1-1-1	13
43	144	Bell			36	I, II	4-8-8-16	440

Time	Rank #	Cond.g Stim.	Disch. Time, sec	Delay Per., sec	Cond.d Refl. Value in scalar units	Digest. Move.t Rcn.	Cond.d Refl. Value aft. every 5 sec in scalar units	Uncond.d Refl. Value aft. 1 min in scalar units
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Experiment # 570 **, Mar. 2, 1955

2h.02m.	151	Bell	20	4	65	I,II	4-14-18-29	468
07	296	Light		1	64		3-15-21-25	465
12	313	199.5-dB tone		3	36		3-6-11-16	461
17	310	Gurgle +		3.5	48		7-11-12-18	482
22	285	Gurgle -		2	42	I, -	2-3-4-3	11
27	152	Bell		2	57	I,II	5-13-16-23	489

* I: first digestive movement reaction (to stimulus), II: second digestive movement reaction (to feeding)

** In pauses, drowsiness

The value of the conditioned reflex to the strongest stimulus, the tone, did not change after the entire period of vitamin B₁ administration. After the cessation of vitamin B₁ injection, the conditioned reflex values returned rapidly to their original values and the phenomenon of hypnotization disappeared.

In the dog Tresor the increase in tone loudness from 66 to 119.5 dB caused a significantly expressed limited inhibition to this strong tone, which appeared as a significant effect on the weak stimulus in the system, touch (see Table 2). Several times after this period, the animal rejected food and sometimes there was a lengthening of the delay period.

Table 4

Tresor; 500 mg Vitamin B₁ Administered

Time	Rank #	Cond.g Stim.	Disch. Time, sec	Delay Per., sec	Cond.d Refl. Value in scalar units	Digest. Move.t Rcn.	Cond.d Refl. Value aft. every 5 sec in scalar units	Uncond.d Refl. Value aft. 1 min in scalar units
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Experiment # 509, Feb. 23, 1955

3h.33m.	296	199.5-dB tone	30	2.5	17	I,II	3-2-2-0-4-6	240
37	534	Touch		4.5	27		1-3-6-5-7-5	229
41	296	Gurgle +		4	23		1-1-0-5-6-10	243
45	273	Gurgle -		4	2	I, -	1-1-0-0-0-10	5
49	297	119.5-dB tone		12	3	I,II	0-0-1-1-0-1	222
53	535	Touch		4	25		1-2-3-5-6-8	220

Experiment # 512, Mar. 2, 1955

4h.16m.	302	119.5-dB tone	30	4	7	I,II	1-2-0-1-1-2	250
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Time	Rank #	Cond.g Stim.	Disch. Time, sec	Delay Per., sec	Cond.d Refl. Value in scalar units	Digest. Move.t Ron.	Cond.d Refl. Value aft. every 5 sec in scalar units	Uncond.d Refl. Value aft. 1 min
4h.20m.	540	Touch	30	2	50	I,II	5-6-6-8-11-14	257
24	299	Gurgle +		2	20		5-1-1-2-3-8	251
28	276	Gurgle -		4	9	-	1-1-1-3-1-2	15
32	309	119.5-dB tone		7	4	I,II	0-1-0-1-1-1	250
36	541	Touch		3.5	35		2-5-5-8-5-10	252

* See note to Table 3

With this background, vitamin B₁ was given to the dog per os daily over 26 days, with milk, in doses of 500 mg. After 10 days of vitamin B₁ administration, some decrease in conditioned reflex value could be noted, as well as a lengthening of the delay period and hypnotic phases - levelling and paradoxical (experiments 509 and 512, Table 4).

In the last period of vitamin B₁ administration, the conditioned reflex decreased more than did the hypnotic phases (levelling, paradoxical, and ultra-paradoxical) and the conditioned reflex reactions became less stable. At times the second digestive movement reaction was absent (experiments 514 and 516, Table 5).

As is evident from Table 2, there was no special effect on limited inhibition in the administration of vitamin B₁. After ending dosage of vitamin B₁, the values of the conditioned reflexes rapidly reached almost to their original level and the phenomenon of hypnotisation disappeared.

Thus, the results of the experiments conducted indicate that giving dogs vitamin B₁ subcutaneously in 100-mg doses for 13 days, 150-mg doses for 11 days, and 500-mg doses for 6 days leads to a weakening of the conditioned reflex reactions, their stability, and appearances of hypnotisation. These

Table 5

Time	Rank #	Cond.g Stim.	Disch. Time, sec	Delay Per., sec	Cond.d Refl. Value in scalar units	Digest. Move.t Ron.	Cond.d Refl. Value aft. every 5 sec in scalar units	Uncond.d Refl. Value aft. 1 min
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Experiment # 514, Mar. 4, 1955

4h.32m.	306	119.5-dB tone	30	4	6	I,II	1-1-0-0-0-4	219
36	544	Touch		3	38		3-4-5-7-8-11	220
40	301	Gurgle +		4	18		2-2-3-4-4-3	221
44	278	Gurgle -		-	0	I, -	-	8
48	307	119.5-dB tone		4	5	I,II	1-1-0-0-1-2	238
52	545	Touch			24		2-2-2-4-6-8	218

Experiment # 516, Mar. 14, 1955

3h.56m.	310	119.5-dB tone	30	8	6	I,II	0-1-0-1-2-2	233
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Time	Rank #	Cond.g Stim.	Disch. Time, sec	Delay Per., sec	Cond.d Refl. Value in scalar units	Digest. Move.t Rcn.	Cond.d Refl. Value aft. every 5 sec in scalar units	Uncond.d Refl. Value aft. 1 min.
4h.00m.	548	Touch	30	17	14	I, -	0-0-0-1-7-6	238
04	303	Gurgle +		4	7	I,II	1-2-0-2-2-0	234
08	280	Gurgle -		3	14	I, -	3-2-2-3-2-2	8
12	311	119.5-dB tone		-	0		-	238
16	549	Touch		3.5	4	I,II	0-0-1-0-3-0	239

* See note to Table 3

changes commences with vitamin B₁ administration per os later, and a significantly high dosage of the vitamin and a longer period of its administration are required for their appearance. After cessation of vitamin B₁ administration, the conditioned reflex activity of the animals rapidly returned to normal.

Vitamin B₁ administration does not have a noticeable effect on limited inhibition caused by the use of a very strong conditioning stimulus.

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ФИЗИОЛОГИЯ

Л. О. ЗЕВАЛЬД

К ВОПРОСУ О ВЛИЯНИИ ВИТАМИНА В₁
НА УСЛОВНО-РЕФЛЕКТОРНУЮ ДЕЯТЕЛЬНОСТЬ СОБАК

(Представлено академиком К. М. Быковым 17 V 1957)

В одной из предыдущих работ ⁽¹⁾ нами было показано, что введение (подкожно) здоровым собакам витамина В₁ в дозах 2 и 5 мг в течение 11—13 дней не вызывало каких-либо заметных изменений со стороны условно-рефлекторной деятельности, а введение в дозах 10 и 20 мг в течение 20 дней приводило к изменениям функционального состояния коры больших полушарий, выразившиеся в ослаблении условнорефлекторных реакций, неустойчивости их, нарушениях силовых отношений и т. п. Введение витамина В₁ в дозах 50 и 100 мг в течение 10 дней не приводило к каким-либо заметным нарушениям функции коры больших полушарий.

Целью настоящей работы было выяснение влияния больших доз витамина В₁ на условнорефлекторную деятельность здоровых собак, и в частности на запредельное торможение, вызванное применением сверхсильного условного раздражителя. Опыты проведены на двух собаках (Узник и Трезор) по методу шидсвых условных рефлексов.

Условные рефлексы у собак были выработаны на раздражителе: звонок, свет, тон (громкостью 66 и 119,5 дБ), бульканье + и бульканье — (дифференцировка). Как Узника, так и Трезора можно отнести к животным с сильным, уравновешенным типом нервной системы. У обеих собак вначале были выработаны условные рефлексы на тон 66 дБ, а затем сразу, без постепенных переходов, на тон 119,5 дБ. На этом фоне были проведены опыты с витамином. Витамин В₁ вводился Узнику путем подкожных инъекций в дозах 100 и 150 мг. Трезору витамин В₁ вводился с молоком, в дозах 500 мг. Результаты опытов приведены в следующих табл. 1 и 2.

Таблица 1

Средние величины условных рефлексов у собаки Узник при введении витамина В₁ подкожно в дозах 100 и 150 мг (в делениях шкалы за 6 дней)

Условные раздражители	При громкости тона 66 дБ (оп. №№ 539—542)	При громкости тона 119,5 дБ (оп. №№ 546—551)	При введении витамина В ₁ по 100 мг (оп. №№ 557—562)	При введении витамина В ₁ по 150 мг (с конца опыта: №№ 565—570)	После прекращения введения витамина В ₁ (оп. №№ 576—581)
Звонок	62	61	56	56	62
Свет	62	54	47	46	55
Тон	44	36	37	34	35
Бульканье+	59	44	46	39	47
Бульканье—	4	8	5	9	6
Звонок	45	49	42	45	50
Сумма	272	244	228	220	249

Как видно из табл. 1, рефлекс на тон громкостью 119,5 дб, по сравнению с величинами рефлексов на остальные раздражители в системе, имеет незначительную величину. Таким образом, усиление громкости тона с 66 до 119,5 дб вызвало развитие нерезко выраженного запредельного торможения на этот тон. Это запредельное торможение оказало свое влияние и на величину рефлексов на свет, и на бульканье+. Соответственно уменьшилась и сумма условных рефлексов. На этом фоне собаке ежедневно в течение

Таблица 2

Средние величины условных рефлексов у собаки Трезор при введении витамина B₁ per os в дозах 500 мг (в делениях шкалы за 6 дней)

Условный раздражитель	При громкости тона 66 дб. (оп. №№ 481—486)	При громкости тона 119,5 дб (оп. №№ 488—489, 492—495)	При введении витамина B ₁ в дозах 500 мг (с 10 дня введения, оп. №№ 507—512)	При введении витамина B ₁ в дозах 500 мг (в конце опыта: №№ 513—518)	После прекращения введения витамина B ₁ (в опытах №№ 519—524)
Тон	29	19	14	10	17
Касалка	51	31	31	22	30
Бульканье +	26	24	23	20	22
Бульканье —	4	4	6	7	4
Тон	20	13	9	5	12
Касалка	35	26	23	19	29
Сумма	161	116	100	76	100

13 дней вводилось по 100 мг витамина B₁. Введение витамина вызвало с 5-го дня его применения некоторое снижение величины условных рефлексов на звонок и свет, по сравнению с величинами этих рефлексов до начала введения витамина (см. табл. 1). Величины рефлексов при ежедневном в течение 11 дней введении витамина B₁ в дозах 150 мг оставались примерно на том же уровне. Но при этом наблюдалось иногда удлинение периода запаздывания, некоторая неустойчивость условнорефлекторных реакций, сонливость животного во время опыта и гипнотические фазы — уравнивательная и парадоксальная (опыты №№ 566 и 570, табл. 3).

Таблица 3

Ушик. Введено 150 мг витамина B₁

Время	№ по порядку	Условный раздражитель	Время отклика, сек.	Период задержки, сек.	Величина условного рефлекса в делениях шкалы	Порядок действия раздражителя	Величина условного рефлекса за каждые 5 сек.	Величина безусловного рефлекса за 1 мин.
в делениях шкалы								

Опыт № 566. 21 I 1955 г.

3 ч. 18 м.	443	Звонок	20	2	42	I, II	5-7-12-18	462
3 ч. 23 м.	292	Свет	20	4	39	I, II	7-11-12-19	442
3 ч. 28 м.	300	Тон 119,5 дб	20	4	19	I, II	2-4-4-9	416
3 ч. 33 м.	306	Бульканье +	20	3	44	I, II	9-11-12-12	—
3 ч. 38 м.	281	Бульканье —	20	3	6	I, II	3-4-4-4	43
3 ч. 43 м.	144	Звонок	20	3	36	I, II	4-8-8-16	470

Опыт № 570. 2 III 1955 г.

2 ч. 02 м.	151	Звонок	20	4	65	I, II	4-14-18-29	468
2 ч. 07 м.	295	Свет	20	1	64	I, II	3-11-21-24	465
2 ч. 12 м.	313	Тон 119,5 дб	20	3	36	I, II	3-6-11-16	461
2 ч. 17 м.	310	Бульканье +	20	3,5	48	I, II	7-11-12-18	482
2 ч. 22 м.	285	Бульканье —	20	2	12	I	2-3-4-4	44
2 ч. 27 м.	152	Звонок	20	2	37	I, II	5-13-16-23	480

* I — первая цифровая запись для реакции (к раздражителю), II — вторая цифровая запись для реакции (к кормушке).

** В скобках — предмет.

Величина условного рефлекса на самый сильный раздражитель — тон за весь период применения витамина В₁ не изменялась. После прекращения инъекции витамина В₁ величины условных рефлексов быстро вернулись к исходным и явления гипнотизации исчезли.

У собаки Трезор усиление громкости тона с 66 до 119,5 дб вызвало возникновение значительно выраженного запредельного торможения на этот сильный тон, оказавшее значительное влияние и на слабый раздражитель в системе — касалку (см. табл. 2). Несколько раз за этот период наблюдались отказы животного от еды и иногда — удлинение периода закармливания.

Таблица 4

Трезор. Введено 5 г витамина В₁

Время	Угол поворота	Условный раздражитель	Время остановки, сек.	Г. период закармливания, сек.	В. период закармливания, сек.	Поведение животного	Величина условного рефлекса за каждые 5 сек.	Величина безусловного рефлекса за 1 мин.
в делениях шкалы								

Опыт № 509. 23 II 1955 г.

3 ч. 33 м.	296	Тон 119,5 дб	30	2,5	17	I, II	3-2-2-0-0-4-6	240
3 ч. 37 м.	534	Касалка	30	4,5	27	I, II	1-3-6-5-7-5	229
3 ч. 41 м.	296	Бульканье +	30	4	23	I, II	1-1-0-5-6-10	243
3 ч. 45 м.	273	Бульканье —	30	4	2	I, —	1-1-0-0-0-10	5
3 ч. 49 м.	297	Тон 119,5 дб	30	12	3	I, II	0-0-1-1-0-1	222
3 ч. 53 м.	535	Касалка	30	4	25	I, II	1-2-3-5-6-8	220

Опыт № 512. 2 III 1955 г.

4 ч. 16 м.	302	Тон 119,5 дб	30	4	7	I, II	1-2-0-1-1-2	250
4 ч. 20 м.	540	Касалка	30	2	50	I, II	5-6-6-8-11-14	257
4 ч. 24 м.	299	Бульканье +	30	2	20	I, II	5-1-1-2-3-8	261
4 ч. 28 м.	276	Бульканье —	30	4	9	—	1-1-1-3-1-2	15
4 ч. 32 м.	303	Тон 119,5 дб	30	7	4	I, II	0-1-0-1-1-1	256
4 ч. 36 м.	541	Касалка	30	3,5	35	I, II	2-5-5-8-5-10	252

* См. примечание к табл. 3.

На этом фоне собаке ежедневно в течение 26 дней вводился витамин В₁ per os с молоком, в дозах по 500 мг. С 10 дней применения витамина В₁ можно было отметить некоторое снижение величины условных рефлексов, удлинение периода закармливания и гипнотические фазы — уравнивательную и парадоксальную (опыты №№ 509 и 512, табл. 4).

В последний период применения витамина В₁ условные рефлексы снижались еще больше, чаще наблюдались гипнотические фазы (уравнивательная, парадоксальная и ультрапарадоксальная) и условнорефлекторные реакции стали менее устойчивыми. Временами отсутствовала вторая пищевая двигательная реакция (опыты №№ 514 и 516, табл. 5).

Как видно из табл. 2, особого влияния на запредельное торможение введение витамина В₁ не оказало. После прекращения дачи витамина В₁

величины условных рефлексов быстро достигли почти исходного уровня и явления гипнотизации исчезли.

Таким образом, результаты проведенных экспериментов показывают, что введение собакам витамина В₁ в дозах 100 мг в течение 13 дней и 150 мг в течение 11 дней подкожно, и в дозах 500 мг в течение 6 дней приводит к ослаблению условнорефлекторных реакций, неустойчивости их, и явле-

Таблица 5

Время	Возраст по порядку	Условный раздражитель	Время отставания, сек.	Период запаздывания, сек.	Величина условного рефлекса в десятичных единицах	Повторная реакция	Наличие условного рефлекса за каждые 5 сек.	Величина базального рефлекса в десятичных единицах
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Опыт № 514. 4 III 1955 г.

4 ч. 32 м.	306	Тон 119,5 дб	30	4	6	1, II	1-1-0-0-0-4	210
4 ч. 38 м.	544	Касалка	30	3	38	1, II	3-4-5-7-8-11	220
4 ч. 40 м.	301	Бульканье +	30	4	18	1, II	2-2-3-4-4-3	221
4 ч. 44 м.	278	Бульканье —	30	—	0	1, —	—	8
4 ч. 48 м.	307	Тон 119,5 дб	30	4	5	1, II	1-1-0-0-1-2	222
4 ч. 52 м.	545	Касалка	30	4	24	1, II	2-2-2-4-6-8	218

Опыт № 516. 14 III 1955 г.

3 ч. 58 м.	310	Тон 119,5 дб.	30	8	0	1, II	1-1-0-1-2-2	233
4 ч. 00 м.	548	Касалка	30	17	14	1, —	1-0-0-1-7-6	238
4 ч. 04 м.	303	Бульканье +	30	4	7	1, II	1-2-0-2-2-0	234
4 ч. 08 м.	280	Бульканье —	30	3	14	1, —	3-2-2-3-2-2	8
4 ч. 12 м.	311	Тон 119,5 дб	30	—	0	1, —	—	238
4 ч. 16 м.	549	Касалка	30	3,5	4	1, II	0-0-1-0-3-0	239

* См. примечание к табл. 3.

ниям гипнотизации. Эти изменения наступают при введении витамина В₁ не с поздней и для проявления их требуются, по-видимому, значительно большие дозы витамина и более длительное время введения его. После прекращения введения витамина В₁ условнорефлекторная деятельность животных быстро возвращается к норме.

Введение витамина В₁ не оказывает заметного влияния на задержанное торможение, вызванное применением сверхсильного условного раздражителя.

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ЦИТИРОВАННАЯ ЛИТЕРАТУРА

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